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Simultaneous optimization of the resolution and analysis time of flavonoids in reverse phase liquid chromatography using Derringer's desirability function

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ABSTRACT

The chemometrics approach was applied for simultaneous optimization of resolution and analysis time of seven flavonoids in reverse phase liquid chromatography. Derringer's desirability function, a multi-criteria decision making method, was used for the evaluation of two different chromatographic performance goals including resolution and analysis time. The selected flavonoids belong to different classes of flavonoids including: flavonols (myricetin, morin, quercetin and kaempferol) flavones (apigenin and luteolin) and flavanones (naringenin). The effect of five experimental factors on a chromatographic response function, formed using two sigmoidal desirability functions, was investigated. The sigmoidal functions were used to transform the optimization criteria, resolution and analysis time, into the desirability values. The factors studied were percentages of methanol, phosphoric acid and THF in mobile phase as well as flow rate and temperature. A rotatable, orthogonal central composite design was used to map the chromatographic response surface and then calculated chromatographic response functions were fitted to a polynomial model. The obtained regression model was characterized by both descriptive and predictive ability ($R^2 = 0.96$). The model was verified, as good agreement was observed between the predicted and experimental values of the chromatographic response function in the optimal condition. The most effective factor on retention of flavonoids was percentage of methanol in mobile phase followed by temperature, flow rate, THF and H_3PO_4 percentages. Optimum condition for separation of flavonoids was as follows: methanol:0.4% phosphoric acid in water:THF (45.3:54.4:0.3, v/v/v) as mobile phase with flow rate of 1 mL min^{-1} at 30 °C. The optimum condition was applied for separation of flavonoids of *Satureja sahendica Bornm.* which is an endemic medicinal plant of Iran.

1. Introduction

Flavonoids are a large group of plant secondary metabolites based on 2-phenylbenzopyrone structure. Based on the degree of oxidation, saturation present in the heterocyclic C-ring and position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety (Fig. 1) these compounds are classified into the flavonols, flavones, flavanones, flavanonols and isoflavones [1]. Flavonoids are well known for their health benefits such as antioxidant and anticancer properties [2,3], metal chelation ability [4], enzyme inhibition [5] and modulation of gene expression [6]. Because of this wide range of properties, a great number of papers have been published concerning the extraction and identification of flavonoids in plant extracts [7,8].

There is no doubt that reverse-phase liquid chromatography (RP-HPLC) plays a central role in separation and determination of flavonoids. However, despite the various reports on the analysis of flavonoids using RP-HPLC, incomplete separation and coelution of these compounds are the main problems when a complex mixture containing many flavonoids is to be analyzed. These problems are important because some coeluting compounds coexist in plants. In order to solve these problems gradient elution is usually used for separation of flavonoids from different classes. But, gradient elution has some important disadvantages such as "ghost" peaks [9] and base line noise [10] that can lead to inaccurate values of peak area and peak height and impede quantitation. Furthermore, the optimization of gradient elution is more complex as more variables influence the selectivity (primarily gradient steepness, initial eluent strength and secondarily dwell volume) compared to isocratic elution [11]. In terms of separation speed, gradient elution is generally considered to be an inherently slower technique than isocratic elution since a widely accepted rule of thumb indicates that the column should be flushed (i.e. equilibrated) with at least 10 column volumes of initial eluent before reliable retention can be obtained in the next run [12]. These limitations of gradient elution necessitate the development of an optimized isocratic mode of RP-HPLC which could be used for separation of flavonoids of different classes.

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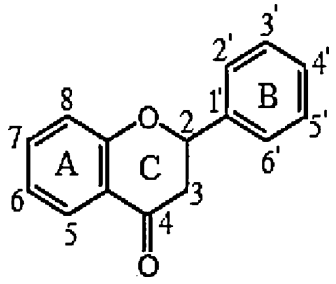

Fig. 1. Chemical structure of flavonoids.

Before any optimization process, two important parameters should be considered. Firstly a criterion should be defined for the evaluation and comparison of the results. The second parameter is the number of experiments. The optimum condition should be obtained with the minimum number of experiments. The selection of the optimization criterion in the field of chromatography may differ from one example to another and the problem becomes more difficult, if one tries to describe the overall resolution in a multicomponent chromatogram [13]. Furthermore, it is usually necessary to judge the very different quality aspects of a chromatogram and to find a compromise between conflicting goals such as maximizing the separation while minimizing the analysis time. Indeed the chromatographic optimization requires a criterion which combines different chromatographic terms, yet allows their simultaneous optimization. The problem of development of such a combined criterion usually called a chromatographic response function (CRF) which consists of a factor related to the time and a factor describing the separation quality [14]. Different approaches from multi-criteria decision making (MCDM) have been used for simultaneous optimization of the criteria in RP-HPLC method [15,16]. Furthermore, due to the dependence on a large number of factors including the mobile phase composition, percentage of organic modifier, flow rate and temperature, optimization of the chromatographic conditions is a complicated process in RP-HPLC. On the other hand, since the effect of these factors on retention can be interdependent and nonlinear the systematic approach for optimization of chromatographic separations is more expedient for such complicated method [17,18].

Recently instead of using traditional one-factor-at-a-time approach for optimization, experimental design is often used for optimization of separation. An experimental design approach is based on the use of matrix experiments which study the simultaneous variation of all operating factors [19]. By application of experimental design one could obtain the maximum information about the optimization process with the minimum number of experiments. The aim of the present work was to develop, optimize and validate a simple isocratic mode of RP-HPLC for simultaneous separation of flavonols (myricetin, morin, quercetin and kaempferol) flavones (apigenin and luteolin) and flavanones (naringenin). In the next step, the obtained optimized condition was used for separation of flavonoids of *Satureja sahendica Bornm.* which is an endemic medicinal plant of Iran. This study is useful for the identification purposes and leads to defining an optimized condition for separation of mentioned flavonoids. In order to achieve this goal, chemometrics protocols of experimental design, response surface mapping and multi-criteria decision making (Derringer's desirability function) were employed. Optimization was performed using a rotatable, orthogonal central composite design (CCD) which is one of the most known designs for the purpose of optimization. The experimental factors studied were: percentages of methanol, phosphoric acid and THF in mobile phase, temperature and flow rate. Chromatographic response function was used to evaluate the chromatograms. Response surface regression method was employed on the results in order to calculate the coefficients relating the effects of the factors to the CRF values. It should be mentioned that the aim of the work was to develop and optimize an isocratic mode of RP-HPLC in order to select the optimum chromatographic conditions for separation of flavonoids of different classes but not to investigate the mechanism of retention of these compounds.

2. Theory

2.1. Chromatographic response function

The most important aspect of the method development in liquid chromatography is the simultaneous optimization of resolution and analysis time. Response surface mapping (RSM) methods are effective optimization tools because the global optimum can be found [20]. The (RSM) methods describe the relationship between the criteria and the experimental variables. Multi-criteria decision making, a branch of operations research, is a useful method that is applied when more than one optimization criterion has to be taken into account. The essence of MCDM is to judge the different quality aspects of a chromatogram individually and quantitatively [21]. A useful method which was applied for compromise between the very different chromatographic goals was utility function. If the purpose of experiments is the optimization of m criteria Y_j (Y_1, ..., Y_m), the utility function for experiment i is the summation of different criterions as follows:

$$U_i = \sum_{j=1}^{m} W_j Y_{ji} \quad (1)$$

In this method the importance of the criteria is expressed by weighting factors $W_j(W_1, \ldots, W_m)$. As can be seen the multi criteria problem is reduced to the single criteria problem U_i. However, despite the wide application of this method the procedure has some important disadvantages such as difficulties in considering the priori weights for all the criteria and unacceptable value of one or more of the criteria. Therefore, in an effort to solve this problem, Harrington [22] proposed to multiply the criteria instead of summing them. According to this method values of criteria should be scaled between 0 (unacceptable) and 1 (optimal). These values are then called desirabilities. This mathematical model was put in general form by Derringer [23]. In this method by using a one-sided or a two-sided transformation, the individual criteria will be transformed into desirability values and values of desirabilities is ranged between 0 and 1 for undesirable and the most desired vales, respectively. One-sided transformation is used when the purpose of experiments is that the response must be as high as possible [24]. The advantage of the Derringer's desirability function is that if one of the criteria has an unacceptable value, then the overall product will also be unacceptable while, with the utility functions, this is not the case [23]. In the next step the overall quality D is calculated by multiplying the desirability values obtained for the different criteria or by using the geometric mean of them [25]. In the next optimization strategies the superiority of sigmoidal transformation to exponential functions for one-sided transformation of different criteria into desirability values was established [13]. In this method, transformation of the resolution values between the neighboring peaks, $R^{p,p+1}$,to desirability values, $S^{p,p+1}$, ranging between 0 and 1 may be performed using the following equation:

$$S^{p,p+1} = \frac{1}{1 + \exp(-b_0 \times R^{p,p+1} + b_1)} \quad (2)$$

The $S^{p,p+1}$ value should be high ($\approx$1) for maximum and low ($\approx$0) for minimum acceptable values of resolution. These limiting conditions determine the values of the parameters b_0 and b_1 in the

equation. In the next step, overall desirability value (f) for the evaluation of the chromatograms in regard to the integral resolution of n analytes is calculated using geometrical average of all individual desirability values of $S^{p,p+1}(p = 1, 2, \ldots, n-1)$.

$$f = \left(\prod_{p=1}^{n-1} S^{p,p+1} \right)^{1/(n-1)} \quad (3)$$

The evaluation of the desirability of the analysis time, g, of the chromatograms may be also performed using a sigmoidal transformation:

$$g = \frac{1}{1 + \exp(b_2 \cdot t + b_3)} \quad (4)$$

where t is a criterion used as a measure of the analysis time. The g value should be high (≈1) for very short and low (≈0) for long analysis time. Calculation of the parameters b_2 and b_3 is done by employing these limiting conditions. Finally, the chromatographic response function is calculated by multiplying the two desirability values f and g:

$$\mathrm{CRF}(f, g) = f \times g \quad (5)$$

As can be easily found, no priori decisions about the weighting factors have to be made in the procedure and the optimization criterion (CRF) is not sensitive to possible changes in the elution order of the components.

2.2. Rotatable and orthogonal central composite design

Central composite design (CCD) is one of the most known designs for the purpose of modeling and optimization. Rotatability and orthogonality are usually considered as the desirable properties of the design. The CCD for f factors consists of N experimental points which are calculated by the following equation:

$$N = N_f + N_a + N_0 \quad (6)$$

where N_f is full factorial or fractional factorial design which is calculated as (2^f) and (2^{f-1}), respectively; N_a is axial experiments carried out on the axes at a distance of $\pm\alpha$ from center of the design and is calculated as ($2 \times f$) and N_0 represents the number of the experiments carried out at the center of the experimental domain. The experiments repeated at the center of the design permit to calculate an independent estimation of the "pure" experimental error variance. The number of the experiments carried out at the center point (N_0) can be calculated by considering the rotatability and orthogonality of the design. A design is rotatable if the variance of the response is constant for all variables at a given distance "α" from the center of the design. Eq. (7) is used to calculate the required α values for rotatability:

$$\alpha = \pm(N_f)^{1/4} \quad (7)$$

In order to determine that among the linear, quadratic and interaction effects of factors which of them are significant, the CCD design should be orthogonal. The rotatable CCD design would be nearly orthogonal if:

$$\alpha^2 = \frac{\sqrt{(N_f + N_a + N_0)N_f} - N_f}{2} \quad (8)$$

3. Experimental

3.1. Chemicals

Standards of naringenin, luteolin and kaempferol, HPLC grade acetonitrile and methanol were purchased from Fluka (Buchs, Switzerland). Quercetin and apigenin were purchased from Aldrich (Milwaukee, WI). Myricetin was obtained from Acros (Geel, Belgium). Morin, THF and phosphoric acid were purchased from Merck (Darmstadt, Germany). Water used was double distilled deionized. All mobile phases were filtered using 0.45 μm filters (Millipore, Bedford, MA, USA).

3.2. Chromatographic conditions

The HPLC system consisted of a model 515 solvent delivery system equipped with model 7725i injector fitted with a 20 μL loop, all from Waters (Milford, MA, USA) and a Perkin-Elmer LC-95 UV detector (Norwalk, CT, USA) set at 260 nm. A Spherisorb C_{18} column (250 mm × 4.6 mm, 5 μm particle size) from Waters was used for all the separations. The column was thermostated at the different temperatures by a water circulator bath. Stock solutions of flavonoids (15.0–30.0 μg mL^{-1}) were prepared in methanol and were stored at 4 °C. The experiments were performed according to the experimental design using a number of eluents prepared with different combinations of the values of the variables. The isocratic chromatographic system was conditioned by passing the eluent through the column until a stable baseline was observed. Then, repeatable retention times were obtained for double subsequent injections. Dead time value was measured from the time of injection of methanol to the first deviation of the base line. All statistical analyses of the response surface regression were performed on range scaled factor values of [−2,+2] with Statistica software.

4. Results and discussion

4.1. Calculation of chromatographic response function

In order to study the application of the desirability function, it was applied to locate the optimum condition for separation of seven flavonoids from different classes with regard to resolution as well as analysis time. To evaluate the quality of the chromatograms using a chromatographic response function, an approach similar to that proposed by Divjak et al. [13] was followed. The resolution between peaks and the retention time of the last peak in the chromatogram were used as the measures of separation and analysis time, respectively. The individual resolution ($R^{p,p+1)}$) between the neighboring peaks p and $p+1$ for n analytes ($p = 1, 2, \ldots, n-1$) was calculated by the following expression:

$$R^{(p,p+1)} = \frac{t_r^{p+1} - t_r^p}{((w^{p+1} - w^p)/2)} \quad (9)$$

where t_r^p, w^p, t_r^{p+1} and w^{p+1} are the retention times and corresponding peak width of two peaks p and $p+1$, respectively. Transformation of the resolution values to desirability values ranging between 0 and 1 was performed using Eq. (2). The minimum acceptable value of the resolution ($R^{p,p+1}$) was set at 0.5 because peaks cannot be recognized as being separated until $R^{p,p+1} = 0.5$. On the other hand, maximum acceptable value of $R^{(p,p+1)}$ was set at 2.5, since higher resolutions resulting in increasing analysis time are of no further benefit. Determination of the parameters b_0 and b_1 in Eq. (2) was done by employing the limiting conditions for values $S^{p,p+1} = 0.95$ and 0.10 for $R^{p,p+1} = 2.5$ and 0.5, respectively. The values obtained for b_0 and b_1 were 2.567 and 3.481, respectively. Then, overall desirability value (f) was calculated using geometrical mean of all individual desirability values $S^{p,p+1}$ (Eq. (3)). The desirability values of the analysis time (g) of the chromatograms were also evaluated using the sigmoidal transformation (Eq. (4)). Calculation of the parameters b_2 and b_3 was done by employing the limiting conditions for values $g = 0.8$ and 0.1 for $t = 30$ and 60 min,

Table 1
Experimental factors, their corresponding five level settings and experimental conditions according to rotatable, orthogonal central composite design of five factors.

Experiment level	X_1 [a]	X_2 [b]	X_3 [c]	X_4 [d]	X_5 [e]
−2	35	0.2	0.5	0.7	25
−1	40	0.3	1	0.8	28
0	45	0.4	1.5	0.9	31
+1	50	0.5	2	1	34
+2	55	0.6	2.5	1.1	37
Fractional factorial design					
1	−1	−1	−1	−1	+1
2	+1	−1	−1	−1	−1
3	−1	+1	−1	−1	−1
4	+1	+1	−1	−1	+1
5	−1	−1	+1	−1	−1
6	+1	−1	+1	−1	+1
7	−1	+1	+1	−1	+1
8	+1	+1	+1	−1	−1
9	−1	−1	−1	+1	−1
10	+1	−1	−1	+1	+1
11	−1	+1	−1	+1	+1
12	+1	+1	−1	+1	−1
13	−1	−1	+1	+1	+1
14	+1	−1	+1	+1	−1
15	−1	+1	+1	+1	−1
16	+1	+1	+1	+1	+1
Central points					
17–26	0	0	0	0	0
Star design					
27	−2	0	0	0	0
28	+2	0	0	0	0
29	0	−2	0	0	0
30	0	+2	0	0	0
31	0	0	−2	0	0
32	0	0	+2	0	0
33	0	0	0	−2	0
34	0	0	0	+2	0
35	0	0	0	0	−2
36	0	0	0	0	+2

[a] Percentage of methanol (v/v, %).
[b] Percentage of H_3PO_4 (v/v, %).
[c] Percentage of THF (v/v, %).
[d] Flow rate (mL min^{-1}).
[e] Temperature (°C).

respectively. The values obtained for b_2 and b_3 were 0.1194 and −4.969, respectively. In the last step, the chromatographic response function was calculated by multiplying the two desirability values f and g (Eq. (5)).

4.2. Experimental design

A full factorial design for five factors and two levels would require 32 experiments. To reduce the number of experiments, a half fractional factorial design (2^{5-1}) with 16 experiments was used. Therefore according to Eq. (6) the number of experiments will be $[(2^{5-1}) + (2 \times 5) + (N_0)]$. According to Eq. (7) the design is rotatable if $\alpha = \pm 2$. Moreover, the design is orthogonal if 10 experiments be carried out at the center point of the design (Eq. (8)). These 36 experiments ($N_f = 16$, $N_a = 10$, $N_0 = 10$) were run in a random manner in order to minimize the effect of uncontrolled variables on the response [26]. Table 1 shows the factor levels used in CCD. The exploration of the experimental domain was started with a factorial design. The experiments 1–16 in Table 1 show the fractional factorial design (fFD). The values of retention times and calculated CRF values for the experiments are reported in Table 2. The reduced design (fFD) allows the first estimation of the effects of the main factors and of their second order interactions that are presented in Table 3. It can be observed that the most important effect on retention time (t_R) values of the flavonoids was due to the percentage of methanol. As expected, an increase in methanol percentage leads to a decrease in retention time. Results showed that the effect of this factor on t_R values is more significant for apigenin and kaempferol. Indeed under the usual reverse-phase conditions, the more polar flavonoids are generally eluted first. Therefore, flavonols are eluted first which followed by flavones and flavanones [27]. Since the pattern of hydroxylation of investigated flavonoids are identical in the A ring the differences in elution pattern is attributed to the OH groups on the B and C rings (Fig. 1). Results showed that the order of elution of compounds is correlated with the number of OH groups on the B and C rings of flavonoids. As the number of hydroxyl groups decreases the polarity of flavonoids decreases and possibility of formation of hydrogen bonding between flavonoids and methanol in mobile phase decreases. This effect leads to stronger retention of flavonoids on the stationary phase. The exception of this statement is naringenin. The number and position of OH groups in naringenin and apigenin are equal (Table 4) but naringenin is the third eluted compound while apigenin is the last one. The only difference in the structure of these two compounds is the absence of double bond in the "C" ring of naringenin. In fact it is established that the absence of a double bond in the "C" ring of naringenin increases the torsion angel of $O–C_2–C_{1'}–C_{6'}$ which leads to an increase in its solubility [28]. Therefore, it seems that the mechanism of solubility of flavonoids is a complicated process and is not only based on the solute–solvent interactions. Further analysis of the results of the experiments of the fFD showed that the most significant effect on CRF values was due to the main factor methanol percentage followed by temperature, flow rate, THF and phosphoric acid (last column in Table 3). Surprisingly, percentage of methanol has a positive effect on the CRF values, although it showed negative effects on the values of t_R. It is established that temperature is an important variable in HPLC, as it has a significant effect on values of t_R. Values of t_R usually decrease with increasing temperature by 1–2%/°C [29]. Furthermore, the acceptance of importance of temperature is based on well-known effects of increase in solute diffusivity [30]. The consequence of increased diffusivity is faster mass transport kinetics, which is reported to result in improvement of column efficiency. As the results of Table 3 show the effect of temperature on the retention time of flavonoids is negative which confirms that t_R values of flavonoids decrease with temperature increasing. As it is expected by increasing the flow rate retention time of flavonoids decreases and just like the effect of methanol, flow rate had a positive effect on the CRF values, although it showed negative effects on the values of t_R. The effect of THF on the retention of flavonoids is negative which indicates that retention time of flavonoids decreases by increasing the THF percentage in mobile phase. This effect must be attributed to the strong polar and/or hydrogen bonding acceptor properties of THF. Furthermore, because of the basic property of THF it can form hydrogen bonding with flavonoids and therefore bring them into the mobile phase and subsequently reduce the retention times. Since flavonoids have slightly acidic properties [31] they will be ionized in mobile phase and interact with residual silanol groups on stationary phase which leads to peak tailing of these compounds. In order to solve this problem acids such as acetic, formic or phosphoric acid are added to mobile phase to reduce the peak tailing of these compounds and hence improve the separation of flavonoids. On the basis of the results of Table 3 increasing the percentage of H_3PO_4 in mobile phase had a positive effect on retention time of flavonoids which means that by increasing the acid concentration retention times are increased. This peculiar effect could be attributed to this fact that the ionization of flavonoids is suppressed by acid and they exist in their neutral form and interact more strongly with alkyl chains of stationary phase and subsequently an increase in their retention times is observed. A further analysis of the results of Table 3 showed that the effect of experimental factors on the retention of flavonoids (with a few exceptions for THF) was in the

Table 2
Experimental retention times (min) and CRF values obtained by the rotatable, orthogonal central composite design.

Experiment	My[a]	Mo	Na	Qu	Lu	Ka	Ap	CRF values
1	13.15	16.76	19.97	23.30	28.79	40.89	46.00	0.07
2	8.17	9.37	11.37	12.62	15.19	20.45	22.84	0.30
3	17.95	21.64	27.07	33.61	41.87	62.08	69.37	0.01
4	6.97	7.95	9.69	10.50	12.38	16.64	18.39	0.20
5	15.07	18.23	23.34	27.83	32.90	50.48	53.40	0.04
6	6.94	7.85	9.64	10.25	11.67	16.15	17.07	0.09
7	13.97	16.75	21.53	25.07	29.45	45.09	47.35	0.05
8	7.99	9.15	11.23	12.30	14.10	19.80	20.96	0.22
9	13.12	15.90	19.85	24.24	30.29	44.85	50.36	0.09
10	6.68	7.71	9.50	10.43	12.43	17.13	18.80	0.22
11	11.46	13.72	17.45	20.75	25.65	37.41	41.71	0.10
12	7.55	8.63	10.48	11.63	13.70	20.56	22.87	0.25
13	12.75	15.39	19.93	23.30	27.30	42.27	44.08	0.12
14	6.11	6.99	8.55	9.41	10.80	15.10	15.92	0.23
15	12.45	15.06	19.28	22.86	26.88	41.98	43.93	0.14
16	5.40	6.05	7.40	7.90	9.00	12.27	12.85	0.10
17	8.80	10.34	12.92	14.63	17.30	24.64	26.69	0.30
18	8.70	10.20	12.75	14.48	17.15	24.50	26.55	0.29
19	8.85	10.34	12.79	14.47	17.10	24.50	26.65	0.30
20	8.68	10.15	12.74	14.46	17.21	24.50	26.52	0.29
21	8.83	10.29	12.78	14.45	17.10	24.49	26.72	0.31
22	8.74	10.23	12.80	14.57	17.23	24.45	26.63	0.32
23	8.73	10.24	12.74	14.42	17.00	24.50	26.65	0.32
24	8.75	10.29	12.89	14.63	17.29	24.55	26.64	0.31
25	8.71	10.19	12.70	14.40	17.02	24.52	26.33	0.32
26	8.76	10.27	12.91	14.68	17.24	24.71	26.82	0.29
27	9.28	10.85	13.55	15.35	18.11	26.20	28.17	0.17
28	10.44	12.36	15.47	17.70	20.89	30.63	32.90	0.22
29	9.49	11.21	13.99	16.11	20.72	29.05	33.27	0.27
30	11.78	14.11	17.44	20.68	24.84	36.32	39.64	0.20
31	8.71	10.15	12.74	14.09	16.00	23.95	24.48	0.20
32	11.15	13.02	16.39	18.37	21.60	30.64	33.32	0.22
33	7.725	9.058	11.666	13.641	16.191	23.00	24.991	0.32
34	27.40	33.74	43.85	55.73	68.67	111.25	120.31	0.00
35	5.27	5.81	6.90	7.33	8.33	10.85	11.70	0.13
36	7.95	9.27	11.70	12.99	15.14	21.56	23.30	0.22

[a] My, myricetin; Mo, morin; Na, naringenin; Qu, quercetin; Lu, luteolin; Ka, kaempferol; Ap, apigenin.

Table 3
The effects of the factors and of their interactions calculated for flavonoids from the fractional factorial design (experiments 1–16 in Table 1).

Factors	Myricetin	Morin	Naringenin	Quercetin	Luteolin	Kaempferol	Apigenin	CRF
X_1	−6.76675	−8.59162	−11.33238	−14.48950	−18.0071	−28.36825	−30.81450	0.1218
X_2	0.216750	0.220875	0.2593752	0.4062503	0.455374	1.064750	1.120750	0.012
X_3	−0.54599	−0.65212	−0.572124	−1.018750	−2.27387	−2.107749	−4.345750	−0.031
X_4	−1.8355	−2.15637	−2.688375	−3.121001	−3.78862	−5.001500	−5.608251	0.0345
X_5	−1.3855	−1.72287	−2.021875	−2.876750	−3.63237	−5.931000	−6.677000	−0.041
X_{12}	−0.21650	−0.25212	−0.326125	−0.504000	−0.68862	−0.9537501	−1.010500	−0.006
X_{13}	−0.187750	−0.25412	−0.482125	−0.308500	0.240624	−0.7557507	0.323000	−0.050
X_{14}	0.7522501	0.920625	1.188625	1.543751	1.934375	3.004499	3.402500	−0.036
X_{15}	0.4272500	0.579125	0.6676248	1.154000	1.553125	2.500500	2.804250	−0.055
X_{23}	−0.483250	−0.57912	−0.765374	−1.070750	−1.29287	−2.280751	−2.466750	−0.019
X_{24}	−0.668750	−0.84987	−1.065625	−1.464500	−1.88212	−2.845500	−3.070750	−0.008
X_{25}	−0.647750	−0.77887	−1.003125	−1.170750	−1.40937	−2.324500	−2.531499	−0.001
X_{34}	0.0230001	0.035125	0.0448749	0.1230000	0.230124	0.023500443	0.1042496	0.0100
X_{35}	0.7440003	0.873125	1.044875	1.404250	1.794875	3.036000	3.460501	−0.028
X_{45}	0.6500000	0.7978751	1.049125	1.435500	1.784625	2.579751	2.764500	−0.000

Table 4
Number and positions of OH groups and double bond in the structure of investigated flavonoids.

Compound	Number of OH groups	Position of OH groups	Double bond in "C" ring
Myricetin	6	3,5,7,3′,4′,5′	Yes
Morin	5	3,5,7,2′,4′	Yes
Naringenin	3	5,7,4′	No
Quercetin	5	3,5,7,3′,4′	Yes
Luteolin	4	5,7,3′,4′	Yes
Kaempferol	4	3,5,7,4′	Yes
Apigenin	3	5,7,4′	Yes

following order:

Apigenin > kaempferol > luteolin > quercetin > naringenin > morin > myricetin

Results of the study showed that the overall CRF value reasonably represented our evaluation of the obtained chromatograms. They gave high values only for the chromatograms that exhibited good separation in a reasonably short analysis time while, medium values were obtained for the chromatograms with relatively good separation but longer analysis time and for the chromatograms with bad separation regardless of the analysis time. The chromatograms that exhibited bad separations in a long analysis time

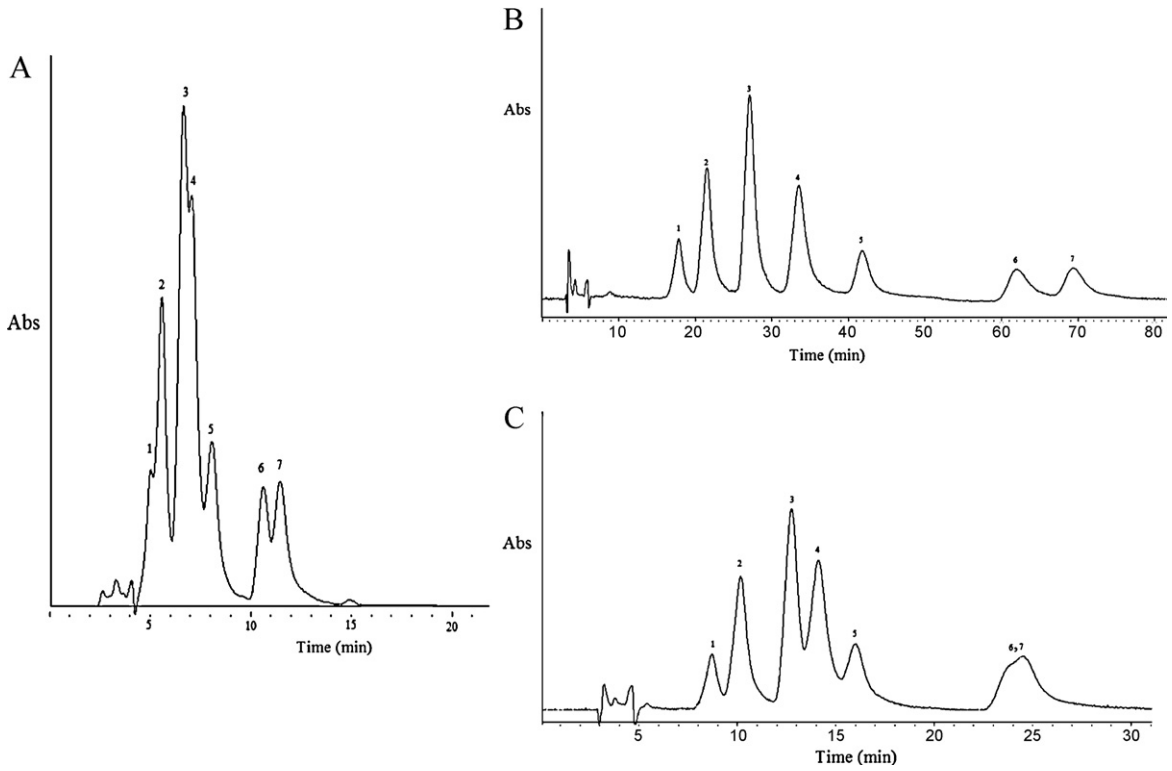

Fig. 2. The chromatograms giving CRF values of 0.13 (A), 0.01 (B) and 0.20 (C). Conditions are as those of the experiments 35, 3 and 31 (Table 2), respectively. Peaks identification: 1, myricetin; 2, morin; 3, naringenin; 4, quercetin; 5, luteolin; 6, kaempferol; 7, apigenin.

had low CRF values. Typical chromatograms with different CRF values are shown in Fig. 2A–C.

4.3. Modeling

The results of the CCD were fitted to the following second-order polynomial model and by applying the response surface regression analysis on the CRF values the regression coefficients (βs) were obtained.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j \quad (10)$$

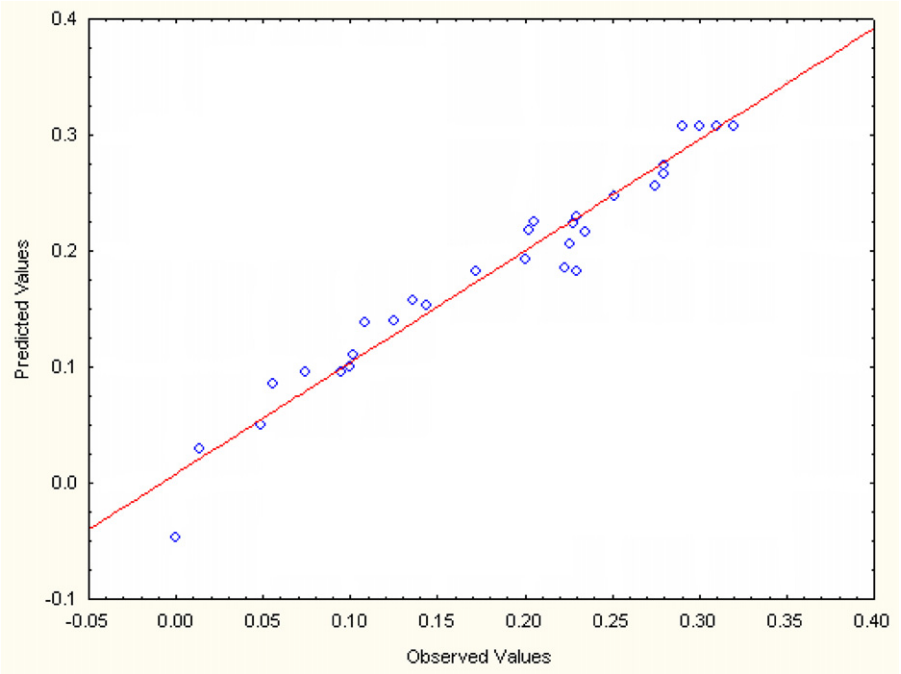

Fig. 3. Plot of predicted CRF values versus experimental values.

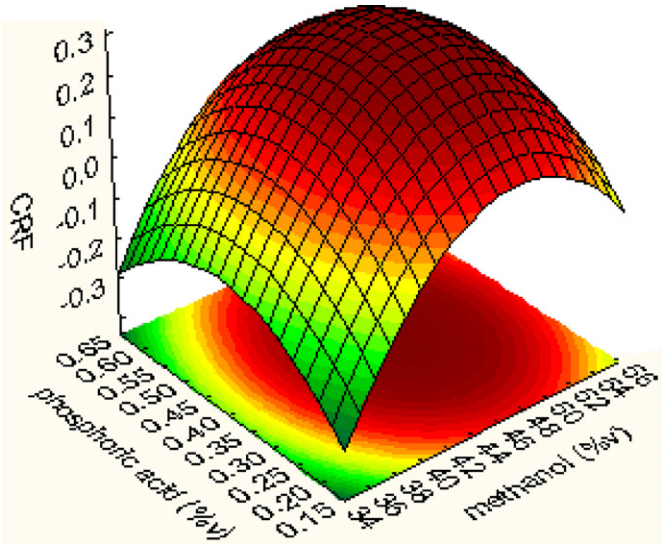

Fig. 4. Response surface plot of CRF versus the volume percentages of methanol and phosphoric acid.

where $x_1, x_2, \ldots, x_k$ are the independent variables affecting the responses (Y); β_0, $\beta_i (i = 1, 2, \ldots, k)$, $\beta_{ii} (i = 1, 2, \ldots, k)$, and $\beta_{ij} (i = 1, 2, \ldots, k; j = 1, 2, \ldots, k)$ are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively and k is the number of variables. The adequacy of the model was evaluated by statistical tests at the considered confidence level ($P = 0.05$). Neglecting the non-significant terms, the final predictive equation is as following:

$$y = 0.3076 + 0.051x_1 - 0.063x_1^2 - 0.030x_2^2 - 0.015x_3 - 0.020x_3^2 + 0.016x_4 - 0.017x_4^2 - 0.010x_5 - 0.025x_5^2 - 0.023x_{13} - 0.016x_{14} - 0.026x_{15} + 0.008x_{23} - 0.015x_{35} \quad (11)$$

Criteria for the evaluation of the descriptive capability of the model were Fisher-ratio value (F) and squared correlation coefficient (R^2). The high values of R^2 and F (Table 5) indicate that the model is quite successful in calculating the chromatographic response function and greater than 96% of the variance is accounted for by the model. In Fig. 3 the plot of predicted CRF values versus experimental CRF is shown which indicates that the model obtained is quite valid and stable as good agreement between the predicted and experimental values obtained. Fig. 4 shows a typical response surface for the design which depicts the response surface plot of CRF versus methanol and phosphoric acid percentages in mobile phase. To find the optimum chromatographic condition for separation of flavonoids, a grid search algorithm written in FORTRAN 77 was used. In this method the factors levels in the form of coded values (−2,0,+2) were applied to the grid framework and the corresponding responses were calculated. Then all the obtained responses were compared with each other and the response with the highest value was considered as the optimum condition. Optimum condition for separation of flavonoids was as follows: methanol:0.4% phosphoric acid in water:THF (45.3:54.4:0.3, v/v/v) as mobile phase

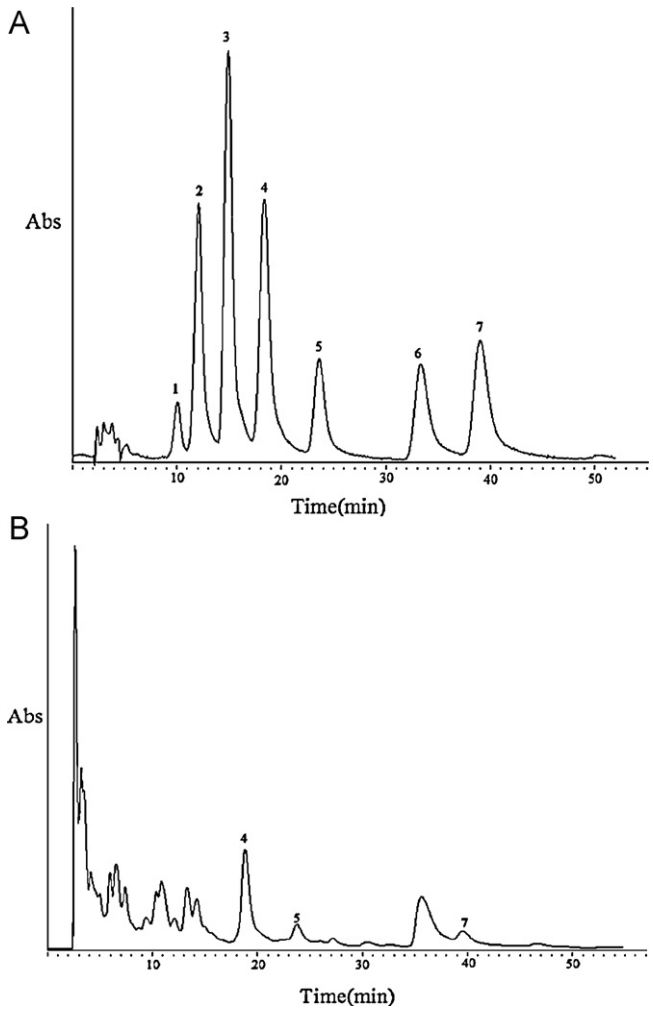

Fig. 5. Chromatograms of (A) standards of investigated flavonoids and (B) extract of *Satureja sahendica Bornm.* at the optimum condition predicted using polynominal model (Eq. (11)). Conditions: 4.6 mm × 250 mm, 5 μm particle size Waters Spherisorb C_{18} column. Mobile phase was methanol:0.4% phosphoric acid in water:THF (45.3:54.4:0.3, v/v/v) as mobile phase with flow rate of 1 mL min^{-1} at 30 °C. Peak identities are as those in Fig. 2.

with flow rate of 1 mL min^{-1} at 30 °C. The efficiency of prediction of the polynomial model was tested by performing the experiment under the predicted optimal condition. The value of predicted CRF (0.34) was in good agreement with the experimental CRF value (0.33) which indicates that the developed model is quite efficient and adequate. Chromatogram obtained under the predicted condition (Fig. 5A) showed adequate resolution of all the flavonoids in a reasonable analysis time. The results of the study demonstrated that it is possible to develop the model with descriptive and predictive ability for the chromatographic response function, which allows one to find the optimum conditions in the separation of flavonoids from different classes. Good resolution achieved in this work permits the identification of the flavonoids from different plants. In order to test the applicability of the obtained

Table 5
Analysis of variance of the regression model.

Source of variation	Sum of squares	Degree of freedom	Mean square	F-value
Regression	0.31176	14	0.022268	37.11
Residual	0.01279	21	0.00060	
Total	0.32455	35		
R^2	0.960			$F_{14/21} = 2.197$

optimum condition it was applied for separation of flavonoids of *S. sahendica Bornm.* which is an endemic medicinal plant of Iran (Fig. 5B).

5. Conclusions

Derringer's desirability function has been introduced to RP-HPLC for optimization of both resolution and the analysis time of flavonoids from different classes. Rotatable, orthogonal central composite design has been shown to be efficient in mapping response surface for changing a chromatographic response function. The results showed that a function composed of two sigmoidal desirability functions can be successfully used to evaluate the chromatograms and to search for an optimum set of experimental conditions. To find the optimal chromatographic conditions, a second order polynomial equation was generated to model the CRF values as a function of the experimental parameters including the flow rate, temperature and percentages of methanol, phosphoric acid and THF in mobile phase. Robustness of the model obtained was assessed using leave-one-out cross-validation method. The efficiency of the prediction of the model was confirmed by performing the experiment under the optimal condition. The results of the study showed that Derringer's desirability function in combination with response surface mapping can be successfully applied to the RP-HPLC separation area for modeling and for process optimization. The method offer promising possibilities in RP-HPC because Derringer approach is the only MCDM method for which is easy to consider simultaneously more than two criteria.

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