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Investigation of Optimum Extraction Conditions for Determination of Quercetin and Kaempferol in Coriander (*Coriundrum sativum* L.) by Using Experimental Design and HPLC

MOHAMMADREZA HADJMOHAMMADI* AND VAHID SHARIFI

Department of Chemistry, University of Mazandaran, P. O. Box 453, Babolsar, Iran

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ABSTRACT

Optimum conditions for extraction of quercetin and kaempferol from coriander are determined using experimental design and HPLC method. Effects of five experimental factors, including percentage of methanol in aqueous extraction solvent, volume of extraction solvent, concentration of HCl used for hydrolysis, extraction time, and temperature on the extraction recovery, are investigated. Central composite design is used to investigate the effects of these factors and grid search method is used to find the optimum conditions. The optimum conditions are 30 mL of 45% aqueous methanol, containing 1.85 M HCl, refluxed for 2 hours at 84°C. The extraction procedures at optimum conditions for quercetin and kaempferol show good repeatability with relative standard deviations of 3.6%, 4.0% and recoveries of about 96%, 94%, respectively. Limit of detection (LOD), Linear dynamic range, intra-day and inter-day R.S.D for quercetin and kaempferol are 0.19, 0.18, 0.19-48.0, 0.18-30.0 μg/mL ($R^2 = 0.99$), and 3.0% - 4.0% respectively. Concentrations of quercetin and kaempferol in Coriander are found to be 23.1 and 10.1 mg/Kg respectively.

Key words: Quercetin, Kaempferol, optimization of extraction, *Coriandrum sativum L,* experimental design, HPLC

INTRODUCTION

Flavonoids are natural products widely distributed in the plant kingdom[1] and are well known for their antioxidant[2], antiviral[3] and anticancer[4] properties. They occur in virtually all parts of the plant, but quantitative distribution varies between different organs of the plant and within different populations of the same plant species. This variability is largely controlled by genetics, maturity, climate and agricultural conditions[5]. Recently there has been a renewed interest in secondary plant metabolites because of their potential preventive effect on the chronic diseases such as cardiovascular disease. Hence isolation, identification and quantification of phytochemicals in foods and evaluation of their potential health benefits have been in focus. However, *in vitro* and animal studies have shown that the action of some chemicals are likely to be achieved only at doses much higher than those obtainable from eating plants[6]. Moreover, knowledge of the precise composition and amount of plant flavonoids may contribute to a better understanding of their influence on the quality and biological properties of these plants[7]. Thus, the extraction of the active ingredient is essential if they are to be of prophylactic or therapeutic value in human subjects[6]. Many factors such as solvent composition, extraction time, extraction temperature[8] and solvent to solid ratio[9], among others, can significantly influence the extraction efficiency. The traditional one-factor-at-a-time approach to process optimization is time consuming and the interactions among various factors may be ignored. Moreover the extraction conditions may not be generalized due to diverse nature of natural antioxidants in different plant materials[8]. Therefore, classical sample handling can decrease quality of the analytical results. In this sense the sample preparation steps account for one-third of the error generated by the analytical method[10]. Instead of using a traditional strategy of optimizing each factor separately, experimental design is often used to select the best conditions for extraction[11-13]. Different methods such as ultrasonic[14], microwave assisted[15], solid phase extraction[16] and pressurized liquid extraction[17] were used for extraction of flavonoids from plants, but solvent extraction procedure is a more broadly used extraction method. Solvents such as methanol, ethanol and acetone or mixtures of these with water are used for extraction[18]. Antioxidants such as butylated hydroxyanisole (BHA), butyl-

* Author for correspondence. Tel: 0112-52-42025-8; Fax: 0112-52-42029; E-mail: Hadjmr@umz.ac.ir

ated hydroxytoluene (BHT) and ascorbic acid are added to extraction solvent to protect the analyte from oxidation[7]. Flavonoids are usually in their glycoside forms in plants. But most reference compounds of these glycosides are not commercially available. Hydrolysis of glycosides to aglycon forms offers a practical method for quantitative determination of flavonoids[19]. Coriander is one of the most consumed vegetables in Iranian diet which its antioxidant activity[20] and the presence of quercetin and trace amount of kaempferol in its extracts have been established[21]. The aim of the present work was to use experimental design to select the optimum conditions for solid-liquid extraction of quercetin and kaempferol from Coriander. This experimental design methodology led us to reduce the number of analysis by choosing informative experiments, which allowed a direct evaluation of the effects of factors involved in the extraction, provided valuable information on the sample treatment procedure, and hence increased the quality of the analytical method. Experiments for the optimization were performed according to the central composite design which is one of the most well known designs for modeling and optimization[22-24]. The experimental factors studied were: percentage of methanol in aqueous extraction solvent (x_1), volume of extraction solvent (x_2), concentration of HCl in extraction solvent used for hydrolysis (x_3), extraction time (x_4) and temperature (x_5). Method of stepwise multiple linear regression (MLR) was employed to select the most important factors and to calculate the coefficients relating these factors to extraction recovery of quercetin and kaempferol. Later the extracted compounds were determined by HPLC – UV.

MATERIALS AND METHODS

I. *Chemicals*

Standards of quercetin and kaempferol were purchased from Sigma (Steinheim, Germany). HPLC grade acetonitrile and methanol were from Fluka (Buchs, Switzerland). Glacial acetic acid, KH_2PO_4, HCl, NaOH and B.H.A (2-tert-butyl-4-methoxyphenol) were from Merck (Darmstadt, Germany). Water used was double distilled and deionized. All mobile phases were filtered using 0.45 µm filter (Millipore, Bedford, MA, USA).

II. *Apparatus and Conditions for Separation and Determination of Quercetin and Kaempferol in Coriandrum sativum L.*

The chromatographic measurements were carried out with HPLC system consisted of a model 515 solvent delivery system equipped with model U6K injector fitted with a 20 µL loop. Column used was Spherisorb C_{18} (250×4.6 mm, 5 µm). All were from Waters (Milford, MA, USA). The UV detector was model LC-95 set at 370 nm from Perkin-Elmer (Norwalk, CT, USA). Adjustment of pH of HPLC mobile phases was done by model 3030 Jenway pH meter (Leeds, UK). The mobile phase used for separation and determination of analytes was mixture of acetonitrile , 0.025 M phosphate buffer (25/75, v/v) with pH = 2.4 and flow rate of 1 mL/min. Identification of each compound was performed by its retention time, spiking with the standard, and comparison of its UV spectrum with the standard. For additional peak identification two other mobile phases were used[25], including water/acetonitrile/glacial acetic acid (70/25/5, v/v/v) and mixture of methanol, 0.025 M phosphate buffer (45/55, v/v) with pH = 2.4. All statistical analyses were performed using SPSS 11 software (SPSS, Bologna, Italy).

III. *Sample Preparation*

Coriander leaves and stems were collected from western Iran, 1900 meter above the sea level (Bijar, Kurdistan province). These samples were dried, pulverized and passed through a no.40 mesh sieve. A volume of 22.5 mL of 60% aqueous methanol (containing 1.5 g/L B.H.A) was added to 1 g of each sample. Then, 7.5 mL of 7.4 M HCl was added to this mixture. Thus the prepared solution consisted of 1.85 M HCl in 45% aqueous methanol (v/v). This solution was refluxed at 84°C for 2 hours with regular stirring before the extract was cooled and filtered to remove solid particles. This solution was filtered by a 0.45 µm filter and 20 µL of it was injected into the HPLC system.

RESULTS AND DISCUSSION

I. *Experimental Design for Extraction of Quercetin and Kaempferol from Coriandrum sativum L.*

Effects of five experimental factors on extraction recovery of quercetin and kaempferol from Coriander were studied. Table 1 shows levels of the selected factors. The exploration of the experimental domain was started

Table 1. Experimental factors for extraction of quercetin and kaempferol from *Coriandrum sativum* L.

	Experimental factors				
Level	X_1	X_2	X_3	X_4	X_5
-	20	10	1	60	50
0	45	30	1.5	120	70
+	70	50	2	180	90

X_1: percentage of methanol in aqueous extraction solvent (v/v %); X_2 : volume of extraction solvent (mL) ; X_3 : concentration of HCl in extraction solvent used for hydrolysis (mol/L); X_4 : extraction time (min); X_5 : temperature (°C).

Table 2. Experimental conditions according to central composite design of five factors for extraction of quercetin and kaempferol from *Coriandrum sativum L.*

Experiment	x_1	x_2	x_3	x_4	x_5	Response (sum of peak areas)
Fractional factorial design						
1	-1	-1	-1	-1	+1	1.55
2	+1	-1	-1	-1	-1	1.00
3	-1	+1	-1	-1	-1	1.65
4	+1	+1	-1	-1	+1	1.20
5	-1	-1	+1	-1	-1	1.85
6	+1	-1	+1	-1	+1	3.88
7	-1	+1	+1	-1	+1	4.10
8	+1	+1	+1	-1	-1	0.86
9	-1	-1	-1	+1	-1	1.35
10	+1	-1	-1	+1	+1	2.72
11	-1	+1	-1	+1	+1	2.93
12	+1	+1	-1	+1	-1	2.68
13	-1	-1	+1	+1	+1	1.35
14	+1	-1	+1	+1	-1	3.80
15	-1	+1	+1	+1	-1	3.99
16	+1	+1	+1	+1	+1	2.60
Central points						
17	0	0	0	0	0	2.40
18	0	0	0	0	0	2.39
19	0	0	0	0	0	2.40
20	0	0	0	0	0	2.40
21	0	0	0	0	0	2.38
22	0	0	0	0	0	2.39
Star design						
23	-1	0	0	0	0	2.15
24	+1	0	0	0	0	2.22
25	0	-1	0	0	0	2.71
26	0	+1	0	0	0	1.88
27	0	0	-1	0	0	2.77
28	0	0	+1	0	0	1.82
29	0	0	0	-1	0	2.91
30	0	0	0	+1	0	2.21
31	0	0	0	0	-1	2.60
32	0	0	0	0	+1	2.32

with a factorial design. A full factorial design for five factors and two levels would require 32 experiments. To reduce the number of experiments, a two-level half fractional factorial (or reduced) design consisting of 2^{5-1} experiments was used. Experiments 1-16 listed in Table 2 show the fractional factorial design and their corresponding responses (sum of peak areas of quercetin and kaempfreol). The reduced design allowed first estimation effects of the main factors and their second order interactions which are presented in Table 3. This table shows that all factors can affect the extraction recovery of these flavonoids. The negative sign of x_1 shows a negative effect on the recovery, since in high percentage of methanol, some lipid components were also extracted, which may limit the extraction of flavonoids[26]. The positive sign of x_2 indicates that an increase in the volume of extraction solvent leads to an increase in the amount of extracted flavonoids, since higher numbers of solvent molecules can interact with solid materials and enhance the extraction. The rate of acidic hydrolysis of flavonoid glycosides depends on the acidic strength of extraction solvent and type of glycosides connected to flavonoids[5]. The most effective factor in the extraction of these two compounds was the concentration of HCl. As shown in Table 3, higher acid concentration increased the recovery of flavonoids, since acid disrupts cell membranes and releases the flavonoids. An increase in extraction time up to 2 hours increases the recovery, but longer extrac-

Table 3. Effects of factors and their interactions calculated from fractional factorial design (Experiments 1-16 in Table 2) for extraction of quercetin and kaempferol from *Coriandrum sativum* L.

Factors	Effect
X_1	-0.15431
X_2	0.33625
X_3	0.94125
X_4	-0.64752
X_5	0.41625
$X_1 \times X_2$	-1.68870
$X_1 \times X_3$	-0.05625
$X_1 \times X_4$	0.57125
$X_1 \times X_5$	0.43375
$X_2 \times X_3$	-0.15625
$X_2 \times X_4$	0.42625
$X_2 \times X_5$	0.02375
$X_3 \times X_4$	-0.40875
$X_3 \times X_5$	-0.03125
$X_4 \times X_5$	-0.94625

tion time had a negative effect on the recovery because of degradation of these compounds[27]. It is accepted that higher temperatures lead to increasing yield of extracted flavonoids[28]. Our results showed that increasing the temperature leads to higher amounts of quercetin and kaempferol recoveries. To estimate the pure experimental error and check system reproducibility, the experiment in the central point was replicated (experiments 17-22 in Table 2). Subsequently, existence of quadratic (or higher) significant effects were tested by means of F-test which compares the difference between the responses at central point and fractional factorial design with the purely experimental variance (S^2_{pe}) according to the following equation[29]:

$$F = \frac{(\bar{y}_0 - \bar{y}_f)^2}{S^2_{pe} \times [(1/n_0) + (1/n_f)]} \quad (1)$$

Where $\bar{y}_0$, $\bar{y}_f$, n_0 and n_f are average response of the replicated central point design, average response of the fractional factorial design, number of central point and number of fractional factorial design experiments, respectively. The high F- value (293) derived from data on Table 2 showed that the quadratic (or higher) effects should be considered in the regression model to describe dependence of the response to the experimental factors. Thus a star design consisting of 10 experiments (experiments 23-32 in Table 2) was done to provide the central composite design to obtain a model containing main factors plus their interactions. The responses obtained from experimental design were subjected to stepwise multiple regression method. Then the model with the most reasonable statistics (higher F and R values and low standard error) was considered as the satisfactory model (Table 4). The model obtained showed that the recovery is influenced by all five main factors and six second order interactions. To find the optimum conditions, a grid search method was used. In this method the dimension of each point in the grid framework (in the form of coded values) was applied and the corresponding responses were obtained. Then all the obtained responses were compared with each other and the response with the highest value was considered the optimum condition. The predicted and experimental responses as well as their optimum levels are shown in Table 5. To evaluate precision of the results obtained by this model, three experiments were carried out under optimum conditions. Results in Table 5 show a good agreement between the calculated and experimental response. The recoveries obtained at optimum conditions (30 mL of 45% aqueous methanol, containing 1.85 M HCl refluxed for 2 hours at 84°C) were 10% higher than those reported by Justesen *et al*[21] (50 mL of 50% aqueous methanol, containing 1.2 M HCl refluxed for 2 hours at 90°C).

II. *Determination of Quercetin and Kaempferol in Coriandrum sativum L. Sample*

Determination of quercetin and kaempferol was performed under the optimum conditions (30 mL of 45% aqueous methanol, containing 1.85 M HCl refluxed for 2 hours at 84°C) using standard addition method with HPLC at λ = 370 nm. Identification of each compound was performed by its retention time, spiking with the standard, and comparison of its UV spectrum with the standards. For additional peak identification, at least three different chromatographic conditions (reported under Table 6) were used[25] to reveal the probability of overlapped peaks. No overlapping peak was observed in chromatograms of Coriander extracts at these conditions. Typical chromatograms of standards of quercetin and kaempferol (A) and Coriander extract (B) are shown in

Table 4. Specification of the best model for prediction of optimum conditions for extraction of quercetin and kaempferol from *Coriandrum sativum* L.

Variable	Coefficient	Standard error	Standardized regression coefficient
Constant	2.358	0.024	-
X_1	-0.285	0.032	0.133
X_2	0.196	0.032	0.172
X_3	0.461	0.032	0.428
X_4	0.335	0.032	0.180
X_5	-0.200	0.032	0.177
$X_1 \times X_2$	-0.664	0.034	-0.582
$X_1 \times X_3$	0.216	0.034	0.189
$X_1 \times X_4$	0.274	0.034	0.240
$X_2 \times X_3$	-0.073	0.034	-0.064
$X_3 \times X_4$	-0.202	0.034	-0.177
$X_4 \times X_5$	-0.473	0.034	-0.415
Statistics			
R^2	0.982		
SE	0.0135		
F	111.428		

R^2: Square of correlation coefficient
SE: Standard error of estimate
F: Fisher-ratio value

Table 5. Predicted optimum and experimental response and their optimum levels

Predicted response	Experimental response	X_1 (v/v, %)	X_2 (mL)	X_3 (mol/L)	X_4 (min)	X_5 (°C)
4.235	4.191	45	30	1.85	120	84

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Figure 1. Concentrations of quercetin and kaempferol in Coriander determined by standard addition method were 23.1 and 10.1 mg/Kg respectively.

III. *Validation of the Determination Method*

Statistical results for validation of determination of quercetin and kaempferol in coriander by refluxed solvent extraction and HPLC are shown in Table 7. Limits of detection (LOD) were calculated on the basis of "$3S_b/m$" where S_b is the standard deviation of blank and is equal to p-p Noise when only mobile phase was passing through the column for 45 minutes and m is the slope of calibration curve**.** Linear dynamic range (LDR) for each compound was performed by plotting the peak area versus concentration. The intra-day precision was determined by analyzing a standard solution in five replicates. The inter-day precision was obtained over 5 days by analyzing the same standard solution. The recovery (accuracy) of the method was confirmed by analysis of the mixtures prepared by adding amounts of flavonoid standards at three concentration levels (5, 15 and 25 μg/mL) to Coriander sample. Recovery of each flavonoid obtained was as follows:

$$\%R = \left[\frac{A_1 - A_2}{A_3}\right] \times 100 \qquad (2)$$

Where A_1, A_2 and A_3 are peak area of extract after addition of standard, pure extract and standard, respectively.

Table 6. Retention times (min) of quercetin and kaempferol at different chromatographic conditions

Compound	t_R[a]	t_R[b]	t_R[c]
Quercetin	12.24	13.01	14.10
Kaempferol	21.70	23.00	24.12

[a] column: Spherisorb C_{18} (250×4.6mm, 5 μm), mobile phase: mixture of acetonitrile, 0.025 M phosphate buffer (25/75, v/v) (pH 2.4).

[b] column: Spherisorb C_{18} (250×4.6mm, 5 μm), mobile phase: water/acetonitrile/glacial acetic acid (75/20/5, v/v/v)

[c] Column: Novapack C_{18} (150×3.9 mm, 4 μm), mobile phase: mixture of methanol, 0.025 M phosphate buffer (45/55, v/v) (pH 2.4).

CONCLUSIONS

The experimental design approach in this study allowed us to determine the optimum conditions for solid-liquid extraction of quercetin and kaempferol from *Coriandrum sativum L,* with the reduced number of experiments. By using this methodology we can overcome the problems associated with traditional sample preparation methods, evaluate the effect of factors on the extraction procedure, and increase the quality of analytical method. Experiments for the optimization were performed according to the central composite design. The most effective factor on the extraction recovery was the concentration of HCL in extraction solvent. Optimum conditions for extraction were 30 mL of 45% aqueous methanol contain-

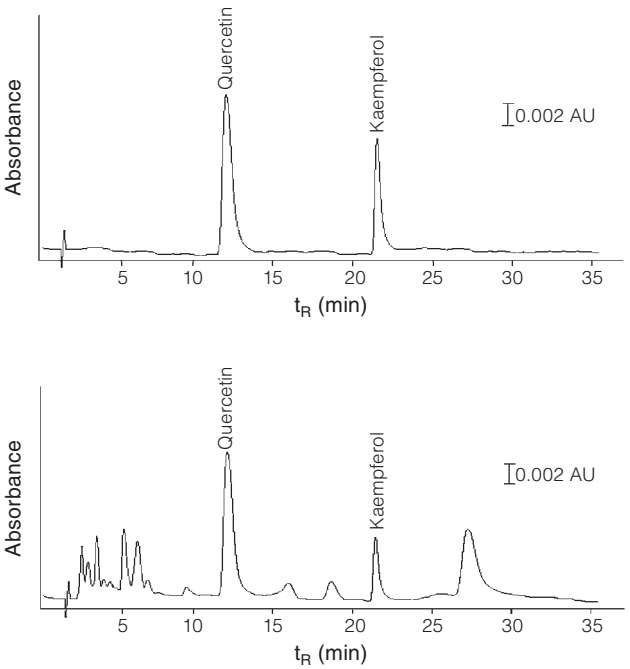

Figure 1. Typical Chromatograms of (A) standard solution of quercetin and kaempferol at the concentration of 20 (μg/mL) and (B) *Coriandrum sativum L.* extract.
Conditions: Column: Spherisorb C_{18} (250×4.6 mm, 5μm); Mobile phase; acetonitrile: 0.025 M phosphate buffer (25/75, v/v) (pH 2.4); Flow rate =1 mL/min; Injection volume = 20 μL; λ= 370 nm.

Table 7. Statistical results for validation of determination of quercetin and kaempferol in *Coriandrum sativum L.* by refluxed solvent extraction and HPLC

Compound	Limit of detection (μg/mL)	Linear dynamic range (μg/mL)	R^2	Intra-day RSD (n = 5)	Inter-day RSD (n = 5)	Recovery (%) (n = 3)
Quercein	0.19	0.19-48	0.997	3.00%	3.24%	96%
Kaempferol	0.18	0.18- 30	0.996	3.45%	4.00%	94%

ing 1.85 M HCl, refluxed at 84°C for 2 hours. There was a good agreement between the predicted optimum and experimental response confirming the validity and adequacy of the predicted model. These extraction conditions showed higher recovery value (10%) as compared with the pervious report [21].

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