Response Surface Methodology for Extraction of Curcumin from Turmeric and Piperine from Black Pepper

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Abstract
Considering that approximately a third of all drugs currently on the market is derived from natural products, curcumin attracted attention due to its anticancer, antioxidative, antiinflammatory and antimicrobial properties. Unfortunately, its low solubility and depending on that a poor bioavailability are limiting factors for its clinical application. It was shown that the administration of curcumin with piperin, the main component of black pepper, increased the bioavailability of curcumin. In addition, piperine increased the plasma concentration and delayed the elimination of drugs like phenytoin and rifampin, and has antiinflammatory and antifungal properties among others. Considering the literature has shown that for extracting curcumin and piperine, respectively, Soxhlet, microwave-assisted extraction, supercritical carbon dioxide extraction and concentional extraction with ethanol as solvent were used among others. According to the concentional extraction of curcumin with ethanol important parameter like the ethanol concentration was not investigated. In addition the maximum extraction time in this case was only 50 minutes, too short in our opinion. Due to these facts the optimum extraction parameters for the conventional extraction of curcumin from turmeric and of piperine from black pepper, respectively, were investigted in this study, with respect to extraction time of 7-21 hours, ethanol concentration of 10-90 % and drug to solvent ratio of 1:10-1:30. Response surface methodology was used as a tool to determine the optimum conditions for the extraction of curcumin and piperine, with help of an experimental design, central composite design. The ideal parameter for this conventional extraction of curcumin from turmeric and of piperine from black pepper, respectively, were an extraction time of 15 hours, an ethanol concentration of 70 % (v/v) and a drug to solvent ratio of 1:20.
Key words: Black Pepper, Curcumin , Extraction, Piperine, Response Surface Methodology, Turmeric.

1. Introduction

Approximately a third of all drugs currently on the market is derived from natural products [1]. The active compound of turmeric (*Curcuma longa*), curcumin (C) [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadi- ene-3,5-dione] has antioxidative [2, 3], anticancer [4, 5], antiinflammatory [6, 7] and antimicrobial [8, 9] properties. A serious disadvantage of C is unfortunately its low solubility and poor bioavailability [9, 10]. This combination of low solubility and poor bioavailability negatively affects its biological efficacy [11] and limits its clinical application. The rapid degradation under neutral or alkaline pH conditions or when exposed to light is another serious disadvantage of C [12]. The orange-yellow powder C is obtained by solvent extraction of ground rhizomes of turmeric.

It is known that co-administration of C with piperin (P) ((2*E*,4*E*)-5-(1,3-benzodioxol-5-yl)-1-(1-piperidinyl)-2,4-pentadien-1-one) is a useful strategy to increase the bioavailability of C [13, 14]. It was postulated that P acts as an apolar molecule forming an apolar complex with drugs and helps to modulate membrane dynamics and the permeability across barriers [15]. P has attracted attention for its antifungal [16], antidiarrhoeal [17], antiinflammatory [18] and 5-lipoxygenase and cyclooxygenase-1 inhibitory activities [19]. In addition, it serves as a positive GABA-A receptor [20, 21]. Several phytochemicals like P, the major component of black

pepper (*Piper nigrum L.*), have been shown to have an activity against P glycoprotein-mediated efflux [22, 23]. P increased the plasma concentration and delayed the elimination of phenytoin [24] and rifampin [25], both P-gp substrates [26, 27]. In addition, the P-gp-mediated transport of digoxin and cyclosporine in the Caco-2 cell monolayers was inhibited by P [28].

The available techniques for the extraction of C are versatile and consist of Soxhlet, microwave-assisted extraction, supercritical carbon dioxide extraction and ultrasonic assisted extraction among others. Soxhlet extraction is time consuming, too laborious and makes the use of bulk amount of organic solvents [29-34]. Several other methods like solid–liquid extraction, aqueous alkaline extraction, extraction with vegetable oils, extraction with aqueous alkanol solutions and supercritical fluid extraction have been developed to extract efficiently compounds from aromatic plants [35]. There are several studies in the literature concerning the extraction of C using different techniques and solvents. In a study of conventionally extraction of C by solvent extraction the extraction temperature (50 - 90°C), the particle size of the turmeric powder (0.42-0.85 mm), the mixing time (10 – 50 minutes) and solvent to meal ratio (10 - 50) were tested be transferring turmeric and a measured amount of absolute alcohol to a flask, which was stirred and heated using a water bath at a selected temperature for a predetermined time. The optimum conditions for the C extraction in this study were 60°C temperature, 0.42 mm particle size, 30 min mixing time and solvent (ethanol) to meal ratio of 50 [36]. For investigating the recovery of C, extracted from turmeric, Microwave-assisted extraction was compared to conventional heat-assisted extraction by varying the solvent concentration (0-100%, v/v), extraction temperature (30-130 °C) and extraction time (0-20 min). Microwave-assisted extraction was performed with acetone, chloroform, ethanol, methanol, and methylene chloride. It could be shown that the extraction of C from turmeric was greatly influenced by the solvent concentration. After adding 20 % water to methanol or ethanol, respectively, the highest extraction yield of C among other solvent mixtures tested was obtained. Increasing the water concentration in the solvent mixtures resulted in a decreased extraction yield of C. There was not a significant difference between the extraction results of 80% (v/v) aqueous methanol and 80% (v/v) aqueous ethanol solutions. C could have an affinity to both polar and non-polar solvents through hydrogen-bonding interactions, although it is a hydrophobic polyphenol having low polarity and low water-solubility [37], leading to formation of H-bonds with solvents like water, methanol and ethanol. The optimum temperature for the extraction of C from turmeric was 80 °C due to the fact that a probably degradation occurred at temperatures higher than 80 °C [38, 39]. The extraction of C was increased with an

increase in extraction time, reached the highest level in 5 min and did not change significantly after this point [40]. In another study C was extracted with polar solvents like isopropyl alcohol, ethyl acetate, acetone by cold and hot extraction. The cold extraction procedure resulted in the higher yield of C [41].

In order to determine the C content in turmeric Soxhlet extraction was used. The investigated parameters were the solvents ethanol (96 %, v/v) and n-hexane, the temperature (25, 40 and 55 °C), the liquid to solid ratio (20, 30 and 40 ml/g) and the extraction time (1, 2.5 and 4 hours) with the help of Box-Behnken Design. It was observed that ethanol was more efficient than n-hexane. The optimum conditions for the C extraction were found as a temperature of 33 °C, a liquid solid ratio of 24 mL/g and an extraction time of 94 min [42].

P is generally isolated from black pepper using volatile organic solvents such as ethanol, toluene or chlorinated hydrocarbons [43, 44]. Using surface-active ionic liquids- aqueous micellar solutions for the extraction of P from black pepper resulted in a strong correlation between extraction yield and the critical micelle concentration of the respective ionic liquid [45]. Conventionally extraction of P is performed by solvent extraction using aliphatic and chlorinated Hydrocarbons like acetone and ethanol for periods of 16- 24 h [43]. It was reported that P was extracted using supercritical carbon dioxide or carbon dioxide + ethanol mixtures [46-48]. In a further study the solvents ethanol, dichloromethane, toluene, heptane and petroleum ether were used for microwave-assisted extraction of P from Piper nigrum resulted in that P was largely extracted in the nonpolar solvents such as petroleum ether [49]. The same authors used in a further study the aromatic sulfonate hydrotropes sodium xylene sulfonate, sodium cumene sulfonate, sodium *p*-toluene sulfonate, *n*-Butyl benzene and sodium butyl monoglycol sulfate for the extraction of P by a continuous Soxhlet extraction with petroleum ether for 48 h. Structural changes in the biomatrix of *Piper nigrum* caused by the solvents lead to an enhanced extraction rate [50]. The combination of the hydrotrope butyl benzene sulfonate with the surfactant sodium dodecyl sulfate in aqueous solutions was investigated for the extraction of P. It was found that beyond the critical micelle concentration of the surfactant the extraction of P increased with an increased concentration of the aqueous SDS solution. The use of butyl benzene sulfonate did not resulted in a significant improved extraction of P [51].

Considering all the versatile techniques and solvents used for the extraction of curcumin and piperine, respectively three crucial decisions had to be made for our study: 1. Choice of extraction technique2. Choice of proper extraction solvent and 3. Choice of effective extraction parameters.

In our case conventional solvent extraction technique was preferred by stirring the turmeric powder and black pepper powder in separate glass beakers for the extraction of both C and P, respectively, due to the fact of being an easy procedure. The literature investigations have shown that for such a conventional extraction like intended here, ethanol was an appropriate solvent. In a study [36] the extraction temperature, the particle size, the extraction time and solvent to solid ratio were tested for the extraction of C. In the present study the extraction temperature was not taken into consideration for two reasons, namely the facts that temperatures of higher than 80 °C are disadvantageous for the extraction of C and the fact that cold extraction procedure results in a higher yield of C [41].

To the best of our knowledge there is no report in the literature about the conventional extraction of P with detailed investigation of the extraction parameters.
Due to these facts it was decided to investigate new parameter like a much longer extraction time of 7-21 hours and ethanol concentrations of 10-90 %, which were not tested before. Furthermore, the drug to solvent ratio as an important parameter in extraction was added to the parameters intended to be investigated.

Response surface methodology (RSM) was used as a tool to determine the optimum conditions for the extraction of C from turmeric and of P from black pepper. RSM is effective for optimizing the process when many factors and interactions affect the desired response [52]. Using a minimum of resources and quantitative data from an appropriate experimental design to determine and simultaneously solve a multivariate equation RSM is an effective statistical method [53]. RSM uses an experimental design likes central composite design (CCD) to fit an empirical model. A CCD combined with a full second-order polynomial model, is very powerful to provides an adequate representation of most continuous response surfaces over a relatively broad factor domain (Deming, 1990). A CCD including independent variables like extraction time, ethanol concentration and drug to solvent ratio was used. Selected response (dependent variable) which evaluates the extraction process was yield of C and P, respectively.

Due to these facts it was aimed in this study to determine the optimum extraction conditions of C from turmeric and P from black pepper, respectively. For that purpose, C and P were extracted by varying the extraction time, ethanol concentration and drug to solvent ratio. Subsequently, the C and P concentrations were simultaneously analysed with HPLC in order to determine out the optimum extraction time, ethanol concentration and drug to solvent ratio.

2. Materials and Methods
2. Materials
The black pepper powder and turmeric powder were supplied from India. Ethanol with analytical grade was purchased from Merck (Merck, Darmstadt, Germany) and the reference substances of C and P were purchased from Sigma-Aldrich (Sigma, St.Louis, MO, USA).

2.1. Analyses of curcumin and piperine with HPLC
The concentrations of C (Figure 1) and P (Figure 2) were analysed with HPLC (Thermo Scientific Ultimate 3000), equipped with a pump and a diode array detector at 262 nm at a column temperature of 33 °C. The mobile phase consisted of a mixture of 0.1 % phosphoric acid and acetonitrile (45:55 %, v/v), and the flow rate was set at 0.8 mL/minute. A C18 column Acclaim® 120 (Thermo Scientific 120 Å C18 3 μm 4.6 x 150 mm) was used, and the injection volume was 20 μL. HPLC assay validation was performed five times a day for 5 consecutive days at a C and P concentration range 0.0625-0.03125-0.01562-0.00675 mg/ml, respectively. This method was modified after [54].
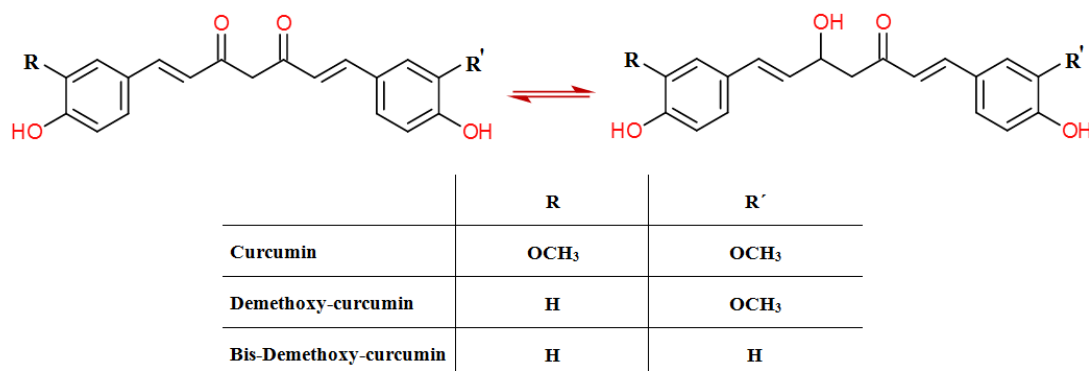

	R	R´
Curcumin	OCH₃	OCH₃
Demethoxy-curcumin	H	OCH₃
Bis-Demethoxy-curcumin	H	H

Figure 1. The chemical structure of curcumin (keto and enol form), desmethoxy-curcumin and bis-desmethoxy-curcumin.
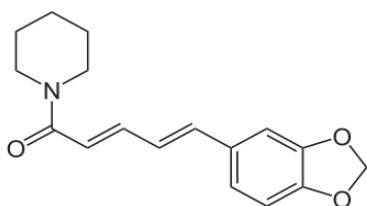
Figure 2. The chemical structure of piperine.

2.2. Extraction experiments for turmeric and black pepper

2.2.1. Curcumin and piperine extraction

The aim of the extraction experiments was to estimate the best possible yield of C from turmeric and of P from black pepper, respectively. Turmeric and black pepper were extracted with selected 20 combinations of parameter (RSM independent variables) such as ethanol concentration (10, 50 and 90 %), extraction time (7, 14 and 21 hours) and drug/solvent ratio (1:10; 1:20 and 1:30 v/w) (Table 1). Turmeric and black pepper powder (each 1 g) were extracted with a magnetic stirrer at 400 rpm in a glass beaker at room temperature. The slurry was filtered through a 0.45 µm polytetrafluorethylene (PTFE) filter (Sartorius, Göttingen, Germany) prior to analysis of the C and P content, respectively, with HPLC. All the experiments were carried out in triplicate.

2.2.2. Experimental design

For determining the influence of three independent variables and the optimum conditions of C and P isolation, a RSM with a CCD was used. The process variables were defined from published data [36, 41, 42, 55, 56]. The response function investigated was C extraction yield y = mg of curcumin/ g turmeric powder and Y = mg of piperine/ g black pepper powder, respectively. The effect of the independent variables x_1 (ethanol concentration, %), x_2 (extraction time, hours) and x_3 (drug to solvent ratio, g/ml) at three variation levels in the extraction process, is shown in Table 1. Six replicates (runs 3,9,10,12,15 and 19) at the centre of the design were used to allow the estimation of a pure error sum of squares. In order to maximise the effects of unexplained variability in the observed responses due to extraneous factors the experiments were randomized. All experiments were carried out in a randomized order to minimize any effect of extraneous factors on the observed responses.

Table 1. Independent variable values of the process and their corresponding levels.

independent variable	symbol coded	Levels		
		-1	0	1
Ethanol concentration (%)	X_1	10	50	90
Extraction time (hours)	X_2	7	14	21
Drug to solvent ratio (g/ml)	X_3	1:10	1:20	1:30

The concentrations of C and P in 70 % aqueous ethanol solution after the extraction experiments were determined with HPLC. Further investigations were made with these concentrations and if necessary with their suitable dilutions.

3. Results and Discussion

3.1. Analyses of curcumin and piperine with HPLC Results and Discussion

A single HPLC method for the simultaneously analysis of C and P was developed, with retention times of 6.2 minutes for C and 6.7 minutes for P (Figure 3).
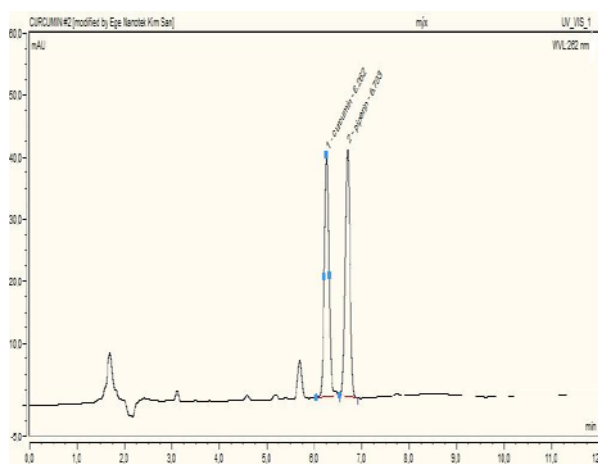

Figure 3. A chromatogram of curcumin and piperine obtained with HPLC.

3.2. Extraction experiments for turmeric and black pepper

The aim of the extraction experiments of turmeric and black pepper was to obtain a high yield of C and of P, respectively. To show the main and interactive effects of the independent variables on the dependent one the 3D surface plots were drawn. The effects of ethanol concentration (10, 50 and 90 %) (Figure 4), extraction time (7, 14 and 21 hours) and drug to solvent ratio (1:10, 1:20 and 1:30) on the response expressed as yield are shown in Table 2 and 4. The response surfaces based on these coefficients are shown in Figure 5 and 6 with one variable kept contant and varying the other two within the experimental range. In general, exploration of the response surfaces indicated a complex interaction between the variables.

The extraction experiments were interpretated with help of the MiniTab optimisation software using the RSM by performing the CCD.

Investigating the C and P yield results revealed the ideal conditions for their extraction as an extraction time of 15 hours, an ethanol concentration of 70% and a drug to solvent ratio of 1:20.
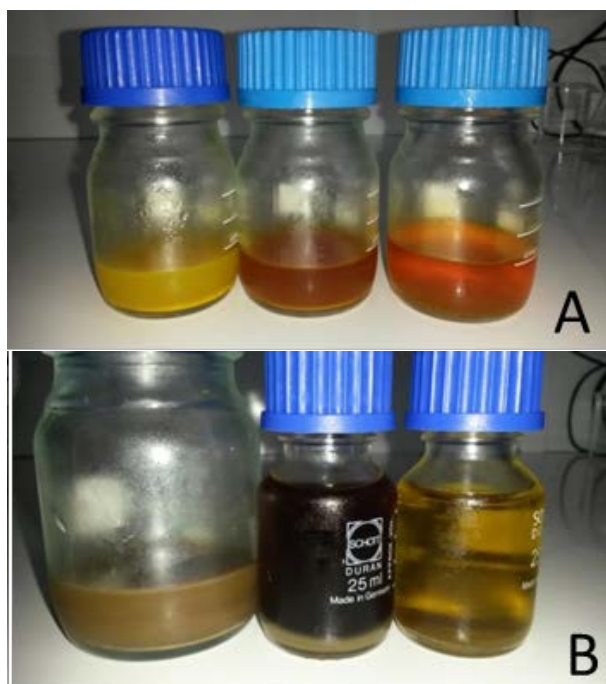
Figure 4. Images of turmeric (A) and of piperine (B) extracts with different ethanol concentrations (10, 50 and 90 %).

Table 2. Experimental design and experimental results of the curcumin extraction from turmeric.

run order	X_1	X_2	X_3	yield (mg curcumin/ g turmeric powder)
1	10	7	30	0.0271
2	90	7	10	6.8770
3	50	14	20	2.6960
4	10	14	20	0.0500
5	90	21	30	4.5783
6	50	14	10	0.7500
7	10	7	10	0.0480
8	10	21	30	0.0090
9	50	14	20	2.6040
10	50	14	20	2.3560
11	50	14	30	2.8250
12	50	14	20	2.4860
13	50	21	20	5.9760
14	10	21	10	0.0170
15	50	14	20	2.3850
16	90	7	30	9.0512
17	90	14	20	4.8120
18	50	7	20	6.2600
19	50	14	20	2.6850
20	90	21	10	7.1300

X_1 = ethanol concentration (%); X_2 = extraction time (hours); X_3 = drug to solvent ratio (g/ml).

The obtained results of the C extraction from turmeric were statistically evaluated by variance analyses (Table 3).

Table 3. Variance analysis results of the curcumin extraction from turmeric.

Source	DF	SS	MS	F	P
X_1	1	104.3112	104.3112	96.13	0.0003
X_2	1	2.073	2.073	1.91	0.197
X_3	1	0.278	0.278	0.26	0.623
$X_1 X_1$	1	1.292	1.292	1.19	0.301
$X_2 X_2$	1	24.772	24.772	22.83	0.001
$X_3 X_3$	1	4.858	4.858	4.48	0.060
$X_1 X_2$	1	2.174	2.174	2.00	0.187
$X_1 X_3$	1	0.015	0.015	0.01	0.908
$X_2 X_3$	1	2.777	2.777	2.56	0.141
Error	10	10.851	1.085		
Total	19	147.890			

DF = degrees of freedom; SS = sum of squares; MS = mean square; F = F value; P = P value

The regression equation (Equation 1) of the curcumin yield, depending on several variables.

$$yield = 1 + 0.1518X_1 - 1.519X_2 + 0.672X_3 - 0.000429X_1X_1 + 0.0613X_2X_2 - 0.01329X_3X_3 - 0.00186X_1X_2 - 0.000109X_1X_3 - 0.00842X_2X_3$$
(Equation 1)

The correlation coefficient R^2 of the Equation 1 is 0.9266. The extraction yield resulting from the turmeric extraction depending on several parameters is given in the 3 dimensional (Figure 5).
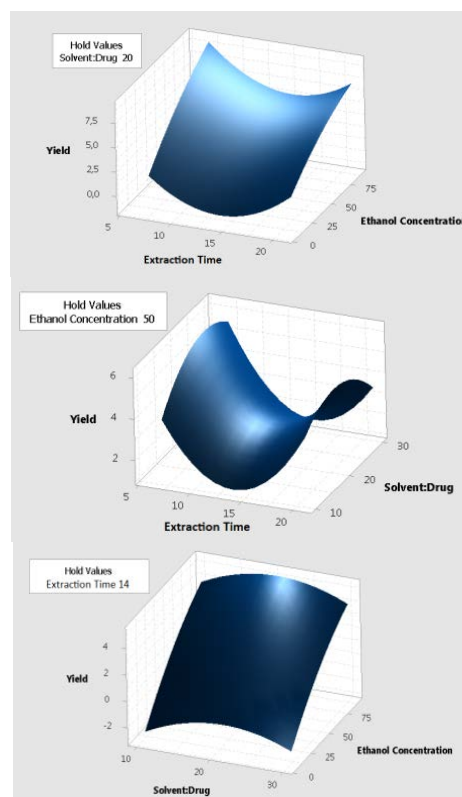

Figure 5. Variation of curcumin yield depending on extraction time and ethanol concentration (a), extraction time and drug:solvent relation (b) and drug:solvent relation and ethanol concentration (c).

Table 4. The experimental design and the experimental results of the piperine extraction from black pepper.

run order	X_1	X_2	X_3	yield (mg piperine/ g blackpepper powder)
1	10	7	30	0.018
2	90	7	10	22.605
3	50	14	20	21.336
4	10	14	20	0.010
5	90	21	30	23.557
6	50	14	10	13.266
7	10	7	10	0.053
8	10	21	30	0.370
9	50	14	20	22.242
10	50	14	20	19.734
11	50	14	30	21.437
12	50	14	20	19.998
13	50	21	20	18.658
14	10	21	10	0.023
15	50	14	20	20.210
16	90	7	30	27.798
17	90	14	20	23.738
18	50	7	20	22.572
19	50	14	20	20.048
20	90	21	10	21.362

X_1 = ethanol concentration (%); X_2 = extraction time (hours); X_3 = drug to solvent ratio (g/ml).

The obtained results of the P extraction from black pepper were statistically evaluated by variance analyses (Table 5)

Table 5. Variance analysis results of the piperine extraction from black pepper.

Source	DF	SS	MS	F	P
X_1	1	1406.26	1406.26	478.18	<0.001
X_2	1	8.24	8.24	2.80	0.125
X_3	1	25.19	25.19	8.57	0.015
$X_1 X_1$	1	155.21	155.21	52.78	<0.001
$X_2 X_2$	1	4.15	4.15	1.41	0.262
$X_3 X_3$	1	11.39	11.39	3.87	0.077
$X_1 X_2$	1	4.21	4.21	1.43	0.259
$X_1 X_3$	1	6.26	6.26	2.13	0.175
$X_2 X_3$	1	0.86	0.86	0.29	0.601
Error	10	29.41	29.41		
Total	19	1812.25	1812.25		

DF = degrees of freedom; SS = sum of squares; MS = mean square; F = F value; P = P value

The regression equation (Equation 2) of the curcumin yield, depending on several variables.

$$yield= -11.95 + 0.7581X_1 - 0.609X_2 + 0.928X_3 - 0.004695X_1X_1 + 0.0251X_2X_2 - 0.0204X_3X_3 - 0.00259X_1X_2 + 0.00221X_1X_3 - 0.00467X_2X_3$$

(Equation 2)

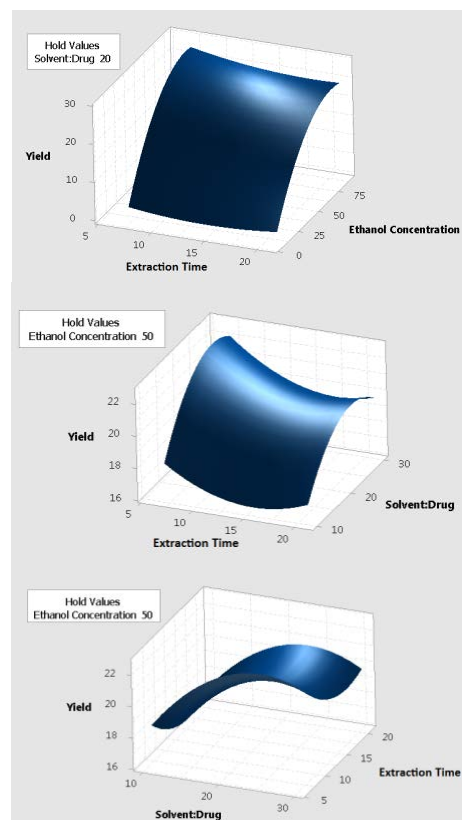The correlation coefficient R^2 of the equation 2 is 0.9838.

Figure 6. Variation of black pepper yield depending on extraction time and ethanol concentration (a), extraction time and drug:solvent relation (b) and drug:solvent relation and ethanol concentration (c).

Investigating the yield results of the extraction experiments of C from turmeric (Figure 5) and of P from black pepper (Figure 6) revealed that after an extraction time of 15 hours no further improvement was determined. The experimental design analysis showed that the variables affecting the extraction yield are the ethanol concentration and drug to solvent ratio. The ideal parameter for obtaining a high yield from the extractions were an extraction time of 15 hours, an ethanol concentration of 70 % (v/v) and a drug to solvent ratio of 1:20.

The concentrations of C and P in 70 % aqueous ethanol after the extraction experiments were determined as 0.49 and 1.21 mg/ml, respectively.

4. Conclusion

In this study, the optimum parameters for the conventional extraction of C from turmeric and P from black pepper were successfuly determined in order to achieve high yields.

For that purpose a HPLC method was successfully developed for the simultaneously analysis of C and P. Several parameters such as, different extraction times, ethanol concentrations and drug to solvent ratios were ivestigated with help of RSM, it was possible to extract C from trumeric and P from black pepper at high yields.

The ideal parameter for this conventional extraction of C from turmeric and of P from black pepper, respectively, were the extraction time of 15 hours, ethanol concentration of 70 % (v/v) and drug to solvent ratio of 1:20.

The concentrations of C and P in 70 % aqueous ethanol after the extraction experiments were determined as 0.49 mg/ml and 1.21 mg/ml with HPLC, respectively.

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