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A Screening Experimental Design to Develop High Extraction Yield of Flavonoids in *Ginkgo biloba* Leaves

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Abstract: Response surface methodology (RSM) was applied to optimize the solvent extraction process of pharmaceutically active flavonoid glycosides from *Ginkgo biloba L*. The three most critical experimental parameters (solvent ethanol content, particle size of the powdered *Ginkgo biloba L.*fragments and the extraction time) were selected in accordance with Plackett-Burman screening design. A mathematical model has been adapted using the RSM coupled with face-centered central composite design (FCCCD) to predict the change in extraction yield of flavonoid components. The regression analysis results indicated the optimal values for reaching the maximum extraction yield of flavonoids from *Ginkgo biloba L*. were 68.22 %, 154.09 μm, and 150.33 minutes for the ethanol content, particle size and extraction time, respectively.

Keywords:*Ginkgo biloba L.*, Flavonoid glycosides, Plackett-Burman screening design, Response surface methodology.

1 Introduction

Flavonoid glycosides, that extracted from natural sources have served as health promoters for many years. *Ginkgo Biloba L*., as one of the most popular origins to these essential phyto components, comprises of the aglycone sources (quercetin, kaempferol and isorhamnetin) to most of these flavonoid compounds, and its medicinal product is currently available in solid oral dosage formulations [1]. Flavonoid components are mostly originated from the aglycones. Some potential novel drug delivery systems for these phenolic components have been previously reported in the literature [2]. The therapeutic action of *Ginkgo Biloba L*. extract, is not only due to its phenolic content. In addition to that the terpene trilactones (terpenoids) present in the extracts also contribute to the pharmacological effect of the extract, such as inhibition of the platelet activating factor(PAF) which is known to magnify neoronal mulfunctioning [3, 4]. To sum up,*Ginkgo Biloba L*. extract has proven potential to: (1) enhance memory, [5, 6] (2) act as a neuroprotective and immune modulatory agent, [7-9] (3) protect against cardiovascular diseases and cancer, [10] (4) possess strong antioxidant activity, [11-14] (5) demonstrate anti-aging effect, [15] (6) effect to increase the blood flow rate, [16] and (7) inhibit the production of nitric oxide (NO) [17].

Several attempts have been reported for the extraction, separation and quantification of the phytopharmaceutical constituents in *Ginkgo Biloba L.* extracts. Besides the conventional solvent extraction (CSE) process, the methods used to extract the therapeutic agents from *Ginkgo biloba L.* vary in a wide range from microwave-assisted extraction to pressurized water extraction (PWE) [18-22]. As the next step forward, the plant extract solution is passed through a column chromatography for detection purposes. The flavonoid glycosides are relatively straightforward to detect and quantify from their aglycone forms.[1,23-27] However, terpene trilactones require the use of some specialized procedures such as, evaporative light scattering detection (ELSD), [28] atmospheric pressure chemical ionization mass spectrometry (APCI-MS),[29] sonic spray ionization source (SSI)-LC/MS technique, [30] and so forth.

The selection of the extraction and separation technique, and further optimization of the protocol is critical to achieve a high recovery yield for the bioactive phytochemicals in the leaves. Numerous factors could be influential in optimizing the production of the plant extract. Therefore, this work aims to successfully satisfy the urgent need for an integrated experimental design-based extraction development study for the flavonoids present in *Ginkgo biloba L.* extracts. A screening experimental design

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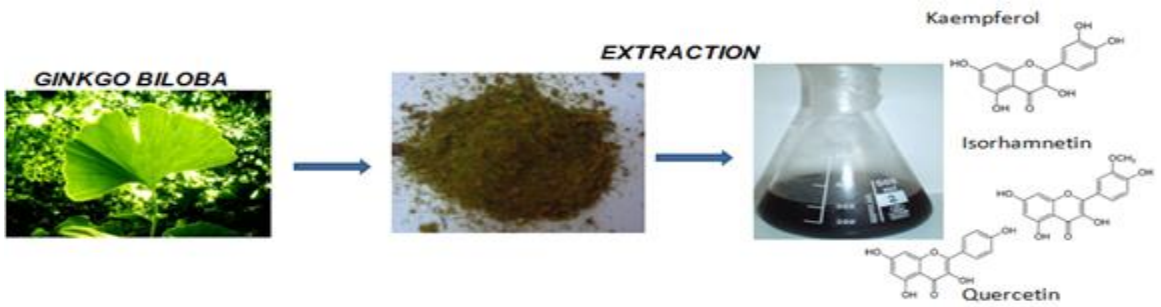

Scheme 1

methodology was adopted to minimize the number of runs required to understand the more important factor effects. Plackett-Burman screening design allows the experimenter to evaluate a large of experimental factors with a very few number of trials and without the need to replicate experiments to draw statistically valid conclusions [31]. To the best of our knowledge, this is the first screening design study for the solvent extraction of *Ginkgo bilobaL.* Such initial screening was followed by response surface methodology (RSM) using face-centered central composite design (FCCD) in order to optimize the extraction yield of the target analytes.

2 Experimental

2.1 Materials

The reference standards of the three flavonoid aglycones-Kaempferol, Quercetin and Isorhamnetin (Fig. 1) were purchased from Fluka (Buchs, Switzerland).

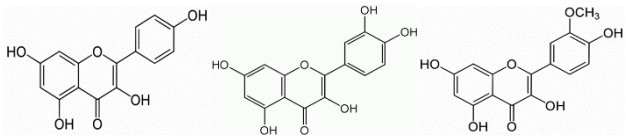

Figure 1 Chemical structure of flavonoid aglycones in hydrolyzed extracts of *Ginkgo biloba L.*

Internal standard, Morin was from Sigma (St. Louis, Missouri). Hydrochloric acid (HCl) (37% purity), methanol, trifluoroacetic acid (TFA) and ethanol were supplied by Merck (Darmstadt, Germany). The solvents used for the chromatographic analysis were HPLC grade and were filtered through 0.2 µm pore size membrane filter (Whatman Inc., Clifton, NJ) prior to flowing through the chromatography column.

The fresh *Ginkgo biloba L.* were collected from a male *Ginkgo biloba* tree (Izmir, Turkey) in the late summer.

2.2 Solvent extraction and hydrolysis

Ginkgo biloba L. were throughly washed with deionized water and dried in an oven for 3 consequtive days at 37°C. The dried leaves were pulvarized with a blender while preventing any possible over-heating of the leaf material [32]. The leaf powder was sieved to different particle sizes, then put in amber glass bottles and stored in a dark place until analysis. Ethanol solution in deionized water was used as extraction solvent. The extraction was performed in 24 hours using a shaking water bath set at 29°C and 200 rpm. The phenolic extract solutions were centrifuged at 3000 rpm for 10 min and then syringe filtered (0.45 µm pore size, Whatman) prior to the hydrolysis step [33].

Transformation of flavonoid glycosides to its aglycones was efficiently achieved by mixing the supernatant solutions with an equal volume of 5.5% HCl in ethanol (v/v) in a shaking water bath for 30 minutes (85°C, 200 rpm) [34]. The hydrolyzed extracts were first filtered through a solid phase extraction cartridge (SPE) (AccuBond, reversed-phase, octadecyl silane packing) to remove the non-polar matrix compounds, and then through a 0.45 µm syringe filter prior to chromatographic analysis.

2.3 Quantification of flavonoid glycosides using reversed-phase high performance liquid chromatography (RP-HPLC)

The chromatographic analysis of the flavonoid aglycones in the hydrolyzed extracts was achieved on a LiChrospher RP-18 column (3250 mm, 5 m packing size) using a gradient elution profile and a mobile phase system consisting of A:water-methanol-TFA (94.95:5:0.05, v/v/v) and B:methanol-TFA (99.95:0.05, v/v) [35,36]. Mobile phase flow rate was kept as 1 ml/min.

The flavonoid aglycone standards were serially diluted to four different concentrations with 80% (v/v) methanol solution in deionized water, and internal standard (morin) was added to each dilution at a concentration of 440 µg/ml [37]. The standard solutions were protected from light until use and syringe-filtered before injecting to the column. The concentration of each aglycone was determined using inline ultraviolet (UV) detection system at 370 nm [38].

Calibration curves were constructed from triplicate readings for each aglycone by taking the peak area ratios of the aglycones to the internal standard for each concentration level. Linear ranges and correlation coefficients are listed in Table 1. The total flavonoid glycoside content of the *Ginkgo biloba L*. extracts was determined according to the study of Hasler et al [39].

Table 1 Linear ranges, regression equations and coefficients for the flavonoid aglycones (n = 3)

Analyte	Linear range ($\mu g\ mL^{-1}$)	Regression equation $y = bx + a^*$	Regression coefficien (R^2)
Quercetin	26 - 500	y = 0.0119x - 0.1698	0.9990
Isorhamnetin	54 - 500	y = 0.0034x - 0.1475	0.9825
Kaempferol	25 - 470	y = 0.02x - 0.2429	0.9969

*y and x stand for the peak area ratio and the concentration of the analytes ($\mu g\ mL^{-1}$), respectively.

2.4 Screening experimental design

Plackett-Burman screening experimental design was adopted in order to draw statistically valid conclusions out of a few selected significant factors from a pool of factors without replicating the experiments [40, 41]. Table 2 shows the screened factors and their experimental range. The screening design required that values for each factor (particle size of leaf material, ethanol content of the extracting solvent, extraction time, solution pH, temperature and shaking rate) be ranged between their low (-1) and high (+1) levels

Table 2 Parameters investigated for Plackett-Burman screening design

Factor	Units	Lower Level (-1)	Higher Level (+1)
A : Leaf particle size	µm	75-150	300-500
B : Solvent ethanol content	%	20	80
C : Extraction time	hour	0.05	6
D : Solution pH	-	1	6.5
E : Temperature	°C	20	45
F : Shaking rate	rpm	20	250

Deciding the experimental range for the parameters involved several considerations, such as the decay of phenolic antixodant activity at high temperatures, change of flavonoid structure in basic solutions, and so forth [42, 43].

The fractional factorial approach deviced by Plackett and Burman reduced the total number of required experiments for the complete full factorial design (64 for 6 parameters) to a relatively quite low number that is 12 [44].Duplicate measurements were conducted for each run in the design. Before starting the 12 run experimental set, five replications of a randomly selected batch was run in order to check for reproducability and to make sure the experimental procedure was rugged. The results of that initial set of experiments was satisfactory.

2.5 Response surface methodology (RSM)

Response surface methodology based on a three-level, three-variable face-centered central composite design (FCCD) was used for optimization of the most significant factors affecting the extraction yield of flavonoid constituents. A total of 18 single replicate runs consisting of four center runs were performed for the selected parameters along with three coded levels (-1, 0, and +1) (Table3).

Table 3 Level and code of variables chosen for FCCD

	Symbols		Coded levels		
Variable	Uncoded	Coded	-1	0	+1
Solvent ethanol content (%)	X_1	x_1	20	50	80
Leaf particle size (µm)	X_2	x_2	112.5	256.25	400
Extraction time (min)	X_3	x_3	2	91	180

In order to find the optimum operating conditions for the selected parameters and characterize the nature of the response surface, the experimental data were fitted to Eq. (1), which is a second order polynomial equation of the form:

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

where y represents the predicted response, β_0 is the intercept term, β_i values linear coefficients, β_{ii} values quadratic coefficients, β_{ij} values interaction coefficients, x_i and x_j represent the level of the independent variables, and k is the total number of variables. Thus, by substituting the value of k and the coded values for each variable, the fitted-model equation takes the form as in Eq. (2):

$$y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (2)$$

The software design expert (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA) was used for the experimental design, data analysis and the quadratic model building. The optimal levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots.

3. Results and discussion

3.1. Screening design study

The use of Plackett-Burman design enabled the identification of relatively significant factors affecting the extraction yield of the therapeutic agents present in *Ginkgo biloba L.* extracts.Table 4 illustrates the extraction yield values of the 12 run screen experimental design for the 6 parameters at their high (+1) and low (+1) levels.

Table 4 Randomized Plackett-Burman design for 6 variables with coded values along with observed results for extraction yield of flavonoids

Run order	Batch	Leaf particle size (A) (µm)	Ethanol content (B) (%)	Extraction time (C) (hr)	Solution pH (D)	Temperature (E) (°C)	Shaking rate (F) (rpm)	Extraction yield (µg/ml)[a]
3	1	+1	+1	-1	+1	+1	+1	1146.06
1	2	+1	-1	+1	+1	+1	-1	1134.51
12	3	-1	+1	+1	+1	-1	-1	1914.13
7	4	+1	+1	+1	-1	-1	-1	1473.10
2	5	+1	+1	-1	-1	-1	+1	969.16
9	6	+1	-1	-1	-1	+1	-1	974.41
10	7	-1	-1	-1	+1	-1	+1	1229.59
6	8	-1	-1	+1	-1	+1	+1	1431.76
11	9	-1	+1	-1	+1	+1	-1	2031.26
8	10	+1	-1	+1	+1	-1	+1	1110.07
4	11	-1	+1	+1	-1	+1	+1	1870.83
5	12	-1	-1	-1	-1	-1	-1	733.51

The factor effects for the design were determined according to the study by Leigh III and Towe, [40] and illustrated in Table 5.

Table 5 Mix Array Showing Test Order and the Specified Level of Each Factor for Each Test Batch

	Batch Formula												Coded Data			
	1	2	3	4	5	6	7	8	9	10	11	12	Sum+	Sum-	Difference	Effect
A	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	-1	6807.3	9211.1	-2403.7	-400.6
B	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	-1	9404.5	6613.8	2790.7	465.1
C	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	8934.4	7084.0	1850.4	308.4
D	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	-1	8565.6	7452.8	1112.8	185.5
E	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	8588.8	7429.6	1159.3	193.2
F	1	-1	-1	-1	1	-1	1	1	-1	1	1	-1	7757.5	8260.9	-503.4	-83.9
E_1	-1	-1	-1	1	-1	1	1	-1	1	1	1	-1	8689.3	7329.1	1360.1	226.7
E_2	-1	-1	1	-1	1	1	-1	1	1	1	-1	-1	8430.8	7587.6	843.2	140.5
E_3	-1	1	-1	1	1	-1	1	1	1	-1	-1	-1	8269.4	7749.0	520.3	86.7
E_4	1	-1	1	1	-1	1	1	1	-1	-1	-1	-1	8169.0	7849.3	319.7	53.3
E_5	-1	1	1	-1	1	1	1	-1	-1	-1	1	-1	8092.6	7925.7	166.9	27.8

According to the same study, the significant factor effect (S_f) and the critical difference (CD) were computed as 128.3 and 258.5, respectively. As a consequence of the analysis of variance, individual factor effects greater than CD ($\geq$ 258.5) were designated as significant. Hence, three factors- particle size of the leaf material, ethanol content of the extraction solvent and extraction time- were found to be significant at the 90% confidence level.

The sign of the individual factor effect indicates whether the effect of the corresponding factor on the extraction yield is positive or negative. For instance, a decrease in the particle size of the leaf material has an inverse effect on the extraction yield, that is it increases the extraction yield. This result is consistent with the fact that smaller particles experience smaller intra-particle diffusion resistance for mass transfer, hence better extraction yields are obtained [45]. On the other hand, an increase in the ethanol content of the extracting solvent and the extraction time increases the extraction yield of the desired constituents.

3.2. Optimization study

The face-centered central composite design (FCCD) matrix of the variables, solvent ethanol content (X_1), particle size (X_2), and extraction time (X_3), is presented in both coded and uncoded units along with the experimental and predicted values of the extraction yield (Table 6).

Table 6 Face-centered central composite design (FCCD) matrix of three parameters in coded and natural units along with the experimental and predicted values of the extraction yield (µg/ml)

	Coded Values			Uncoded Values[a]			Extraction yield (µg/ml)	
Run order	x_1	x_2	x_3	X_1	X_2	X_3	Experimental	Predicted
1	0	0	0	50	256.25	91	1579.91	1601.14
2	-1	-1	-1	20	112.5	2	801.35	905.79
3	1	0	0	80	256.25	91	1649.3	1654.13
4	0	0	1	50	256.25	180	1617.59	1666.32
5	0	0	-1	50	256.25	2	1493.92	1429.56
6	1	1	-1	80	400	2	941.92	1033.32
7	0	0	0	50	256.25	91	1615.06	1601.14
8	-1	1	-1	20	400	2	704.14	638.20
9	0	1	0	50	400	91	1264.29	1269.91
10	1	1	1	80	400	180	1440.51	1339.97
11	-1	-1	1	20	112.5	180	1160.14	1072.65
12	0	-1	0	50	112.5	91	1697.22	1675.97
13	0	0	0	50	256.25	91	1569.67	1601.14
14	0	0	0	50	256.25	91	1608.64	1601.14
15	1	-1	-1	80	112.5	2	1698.71	1633.16
16	-1	1	1	20	400	180	790.93	860.38
17	-1	0	0	20	256.25	91	1071.11	1050.65
18	1	-1	1	80	112.5	180	1814.63	1884.48

Each trial was performed as single replicates due to the previously proven ruggedness of the experimental procedure with the very small replication standard deviation of the four center points. Table 6 shows that the maximum extraction yield for the flavonoid aglycones, 1814.63 µg/mL was achieved in 180 min extraction time at 112.5 µm leaf particle size with 80% solvent ethanol content.

The obtained data were analyzed based on Eq. (3), and the predicted response y was obtained and given below regardless of the significance of the coefficients:

$$y = 1601.14 + 301.74\,A - 203.03\,B + 118.38\,C - 248.75\,A^2 - 128.20\,B^2 - 53.20\,C^2 - 83.06\,AB + 21.12\,AC + 13.83\,BC \quad (3)$$

where, y is the predicted response variable, the extraction yield of flavonoid constituents, and A, B and C are the coded values of the parameters – solvent ethanol content

(%), leaf particle size (μm) and extraction time (min), respectively.

The analysis of variance (ANOVA) for response surface quadratic model of Eq. (3) is summarized in Table 7.

Table 7 Analysis of variance for the fitted quadratic polynomial model of the extraction yield*

Source	Sum of Squares	d.f.	Mean square	*F*-value	Probability *(P)*
Model	2.138×10^6	9	2.375×10^5	29.46	< 0.0001
Pure error	1446.48	3	482.16		
Correlation total	2.202×10^6	17			

* R^2 = 0.9707, R = 0.9852, adequate precision = 18.621.

It is evident that the model is highly significant with the model *F* value (29.46) and a very low probability value (P_{model}< 0.0001). The value of determination coefficient R^2(0.9707) for Eq. (3) shows that the sample variation of 97.1% for the extraction yield is due to the independent parameters and also suggests that the model equation is satisfactory and practicable. The multiple correlation coefficient R (0.9852) is close to 1, indicating the close proximity between the experimental and predicted values [46]. The adequate precision, a measure of signal to noise ratio for the analysis, is 18.621 (ratios > 4 are desirable) and demonstrates the polynomial quadratic model is adequate.

The regression coefficients of Eq. (3) and their significance are presented in Table 8.

Table 8 Regression coefficients and their significance of the quadratic model of the extraction yield

Model term	Coefficient estimate	Standard error	*F* value	Probability > *F*
Intercept	1601.14	35.33	-	-
A - ethanol content	301.74	28.40	112.92	<0.0001
B - particle size	-203.03	28.40	51.12	<0.0001
C – extraction time	118.38	28.40	17.38	0.0031
A^2	-248.75	54.55	20.79	0.0018
B^2	-128.20	54.55	5.52	0.0467
C^2	-53.20	54.55	0.95	0.3580
AB	-83.06	31.75	6.85	0.0308
AC	21.12	31.75	0.44	0.5247
BC	13.83	31.75	0.19	0.6745

The (Probability >*F*) values smaller than 0.05 indicates the significance of the corresponding coefficient. It can be seen from the Table 8 that all linear coefficients (*A*, *B* and *C*) and the quadratic coefficient (A^2) are highly significant, while the quadratic coefficient (B^2) and the interaction coefficient, *AB* are significant.

Towards the goal of determining the optimal values of each parameter for maximum flavonid extraction yield, 3D response surface plots and their respective 2D contour plots were generated.

Fig. 2 depicts the response surface and contour plots of the combined effect of ethanol content of the extracting solvent and particle size of leaf powder on the extraction yield while the remaining variable, i.e., extraction time was fixed at its zero level, 91 min. As deduced from the figure, at low ethanol content values, the extraction yield of flavonoid aglycones decreased gradually with an increase in the particle size of the leaf powder, but decreased sharply with that at higher level of ethanol content. Shown also in the plot was that an increase in solvent ethanol content had more profound effect at relatively small particle sizes.

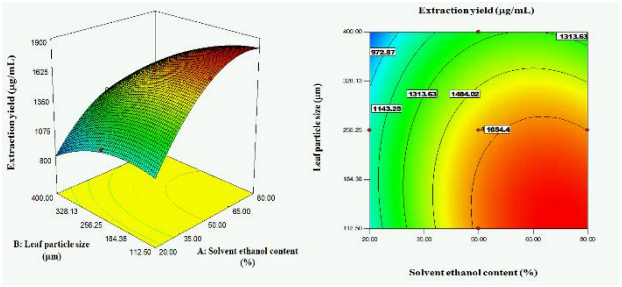

Figure 2 Response surface and contour plots showing the interaction effect of solvent ethanol content and leaf particle size on extraction yield (extraction time = 91 min).

The response surface analysis for the interaction between the extraction time and solvent ethanol content at fixed leaf particle size is presented in Fig. 3.

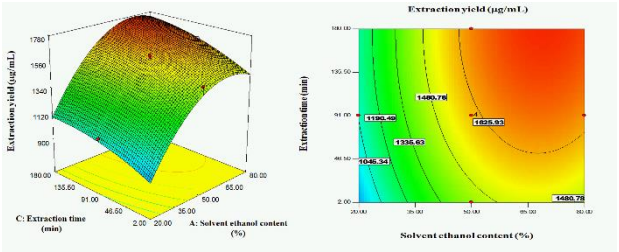

Figure 3 Response surface and contour plots showing the interaction effect of extraction time and solvent ethanol content on extraction yield (leaf particle size = 256.25μm).

The extraction yield of flavonoid glycosides demostrated a gradual increase with an increase in the extraction time, and higher yields were achieved with increased ethanol content. However, solvent ethanol content revealed a proportional relationship with flavonoid recovery up to a point after which the yield could not increase further with increasing ethanol content and started to decay.

Fig. 4 presents the effect of extraction time and leaf particle size on flavonoid glycoside recovery while the solvent ethanol content is fixed at its middle level. The extraction yield tended to gradually increase with extraction time. In

additon, a conspicuous reduction in extraction yield was seen with increasing leaf particle size.

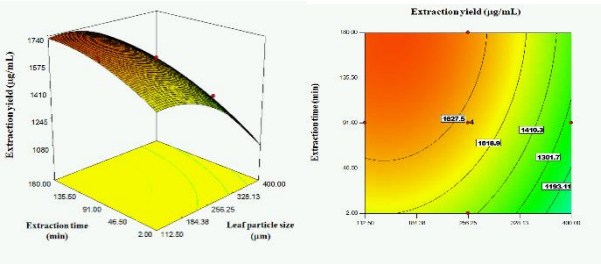

Figure 4 Response surface and contour plots showing the interaction effect of extraction time and leaf particle size on extraction yield (solvent ethanol content = 50 %).

3.3. General interpretation and discussion

Statistical modeling and analysis has been an effective strategy in the optimization of the relatively significant experimental parameters. Prior screening of a pool of factors helps the experimenter focus on more relevant influencing factors. Further analysis of the screened factors using response surface methodologies (RSMs) provides the optimum levels for the combined factors in order to achieve maximum design performance.

To the best of our knowledge, this study is the first to report on the optimization procedure of the conventional solvent extraction conditions of *Ginkgo biloba L.* However, some previous optimization attempts have been outperformed for this plant material utilizing different extraction techniques. Milosevic *et al* [47] optimized the supercritical fluid extraction (SFE) conditions for *Ginkgo biloba L*. using the response surface methodology, and found that the optimal yield was obtained in 3.86 hr at 52.7°C and 184.4 bar. In another optimization study conducted during the accelerated solvent extraction (ASC), it was concluded that the best recoveries were obtained at 100°C with acetonitrile as solvent [48].

Although response surface methodology was only applied for statistical optimization of the extraction parameters in this study, a similar approach could have been applied during the earlier stage of the process. In an earlier study performed by Ji and coworkers,[49] the HPLC fingerprint of *Ginkgo biloba* extracts was significantly improved via screening of the chromatographic separation parameters followed by gradient optimization. Therefore, the optimal extraction yield achieved with this study can be further enhanced with the introduction of optimized chromatographic separation conditions.

The graphical representation through response surface analysis aids the experimenter to visually decide on the optimal values while maximizing the response, which in our case is the extraction efficiency. For instance, it was noticed from this study that the extraction yield of flavonoids tended to increase with increasing ethanol content. However, when the ethanol content was increased further above ~70%, the flavonoid recovery started to decrease significantly. In a similar finding reported by Friedman and coworkers,[50] it was observed for the tea extracts that the recovery rates were lower at high ethanol concentrations. As a concluding remark, this result did not only influence the determination of the optimal ethanol content for the maximal outcome, but also high possibly could contribute to the economical aspects of the process.

These data demonstrate that the optimized conditions are necessary for the extraction conditions of Ginkgo biloba-originated flavonoids. The response surface methodology adopted in this study indicated that the best optimal values for flavonoid extraction were obtained when the solvent ethanol content was close to 70% and the extraction time was around 150 min at a leaf particle size of ~154 µm. These results are expected to have an important impact in the design of efficient statistical models for the other traditional medicinal plants.

4. Conclusions

It is evident from this study that the Plackett-Burman design has proved effective for screening the extraction parameters of *Ginkgo biloba L.*. Further refinement of the best combination of the screened factors particle size of the leaf material, ethanol content of the extraction solvent and extraction time was made through the use of experiments designed to reveal the response surface of the system, that is the mathematical relationship between the screened factors. FCCCD provided relatively precise predictions over a broad area around the center point.

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