An Experimental Design for the Analysis of 5-Caffeoylquinic Acid (5-CQA) in Ethanolic Extracts of Hibiscus (*Hibiscus sabdariffa* L.) Flower

Kísérleti terv a hibiszkusz (Hibiscus sabdariffa L.) virág etanolos kivonatában található 5-koffeoil-kinsav (5-CQA) elemzésére

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Abstract: This study optimized the extraction and analysis of 5-caffeoylquinic acid (5-CQA), the predominant chlorogenic acid in hibiscus flowers, using a sustainable ethanolic extraction method combined with high-performance liquid chromatography (HPLC) analysis. To identify the optimal solvent extraction conditions for 5-CQA from hibiscus, Response Surface Methodology (RSM) was employed, incorporating three factors: pH levels (2, 4.5, 7), extraction times (20, 40, 60 minutes) and solvent compositions (25v/v%, 50v/v%, 75v/v% ethanol). The temperature was maintained at the boiling point throughout the extraction period. RSM identified 25% ethanol, pH 7, and 25-minute heat treatment as the optimal extraction conditions for maximizing 5-CQA yield. Two HPLC columns; InertSustain Phenyl (A) and Synergi Polar (B), differing in length, diameter, and particle size were evaluated for chromatographic performance monitoring HPLC-UV signals at a wavelength of 325 nm. The mobile phase for both columns consisted of acetonitrile and acidified water (with orthophosphoric acid). Both HPLC columns achieved high correlation coefficients (0.9908 and 0.9965, A and B) between extracted 5-CQA and standard peaks. Under optimized conditions, the yields of 5-CQA were 1.43 mg/g and 1.48 mg/g dry hibiscus flower for columns A and B, respectively. Compared to literature-reported methods, the developed protocol offers high efficiency, reduced solvent usage, and minimal heat treatment, establishing it as a convenient, and sustainable alternative for 5-CQA extraction.

Keywords: *5-caffeoylquinic acid; chlorogenic acid; ethanolic extract; hibiscus; HPLC-PDA*

Összefoglalás: Ez a tanulmány optimalizálta az 5-koffeoil-kinsav (5-CQA), a hibiszkuszvirágokban a domináns klorogénsav extrakcióját és elemzését, fenntartható etanolos extrakciós módszerrel kombinálva nagy teljesítményű folyadékkromatográfiás (HPLC) elemzéssel. A hibiszkuszból származó 5-CQA optimális oldószeres extrakciós körülményeinek meghatározásához Response Surface Methodology (RSM) módszert alkalmaztunk, amely három tényezőt tartalmazott: pH-szint (2, 4,5, 7), extrakciós idő (20, 40, 60 perc) és oldószer összetétele (25 v/v%, 50 v/v%, 75 v/v% etanol). A hőmérsékletet a forrásponton tartottuk az extrakciós periódus alatt. Az RSM a 25%-os etanolt (pH 7) és a 25 perces hőkezelést azonosította optimális extrakciós körülményként az 5-CQA hozam maximalizálásához. Két

HPLC oszlop; A hosszúságban, átmérőben és részecskeméretben eltérő InertSustain Phenyl (A) és Synergi Polar (B) kromatográfiás teljesítményét a HPLC-UV jelek 325 nm-es hullámhosszon történő monitorozására értékelték. Mindkét oszlop mozgófázisa acetonitrilből és (ortofoszforsavval) savanyított vízből állt. Mindkét HPLC oszlop magas korrelációs együtthatót ért el (0,9908 és 0,9965, A és B) az extrahált 5-CQA és a standard csúcsok között. Optimalizált körülmények között az 5-CQA hozama 1,43 mg/g és 1,48 mg/g száraz hibiszkuszvirág volt az A és B oszlopban. Az irodalomban közölt módszerekhez képest a kifejlesztett protokoll nagy hatékonyságot, csökkentett oldószerhasználatot és minimális hőkezelést kínál, így kényelmes és fenntartható alternatívát jelent az 5-CQA extrakcióhoz.

***Kulcsszavak**: 5-koffeoil-kinsav; klorogénsav; etanolos kivonat; hibiszkusz; HPLC-PDA*

1 Introduction

There is a whole family of chlorogenic acids (quinic acid esters with substituted cinnamic acids) generated from substitutions at the aromatic ring and the isomers and epimers in the cyclohexane part (Mok et al., 2022; Mukherjee et al., 2015; Sakai et al., 2022). The three major classes of chlorogenic acids are caffeoylquinic acid (CQA), dicaffeoylquinic acid (diCQA), and feruloyl quinic acid (FQA), and the three most available CQA isomers in plant source are; neochlorogenic acid (3-O-caffeoylquinic acid, 3-CQA), crypto chlorogenic acid (4-O-caffeoylquinic acid, 4-CQA), and chlorogenic acid (5-O-caffeoylquinic acid; 5-CQA). The most abundant among them is 5-CQA, a major polyphenol found in many plant sources such as green coffee beans, hibiscus, honeysuckle, and so on and is extensively studied because of its tremendous health benefits (Aree, 2023; Grujic et al., 2015). For instance, 5-CQA has received the attention of scientific communities mainly because of its antioxidant, anti-inflammatory, antibacterial, anti-cancer, and analgesic activities as well as beneficial regulatory effects caused on gut microflora. Hence, regular consumption of a diet rich in 5-CQA is known to reduce the occurrence of many non-communicable diseases (Bhandarkar et al., 2019; Plazas et al., 2013).

The calyces and leaves of *Hibiscus sabdariffa* L., also known as hibiscus and roselle, which belongs to the Malvaceae family are popular in tropical regions, especially in countries in Asia, South America, and Australia, for manufacturing herbal tea due to their therapeutic effects. Additionally, hibiscus calyces have been used as coloring, and flavoring agents in foods, medicines, and cosmetics (Chongwilaikasem et al., 2024). The aqueous and organic extracts of hibiscus calyces are rich in bioactive compounds including flavonoids, antocyanidins and chlorogenic acids. For instance, in a study by Chongwilaikasem et al (2024), the ethanolic extracts of hibiscus calyces have demonstrated moderated DPPH radical scavenging ability (EC_{50} = 289.61µg/mL) and strong antibacterial effects against several bacterial species including *Escherichia coli* and *Staphylococcus aureus*. Hence, hibiscus floral extracts have recently gained the attention of scientists in their uses in functional food applications (Paraı´so et al., 2019).

Although several studies are focusing on the overall bioactivity of hibiscus flower extracts and anthocyanin extraction from hibiscus flower, studies focusing on extraction of 5-CQA from hibiscus flower are minimal. The extraction process plays an important role in preserving the bioactivity and the food applications of the compounds of interest. For instance, the conventional extraction methods utilize large quantities of organic solvents which become toxic in food applications and increase the extraction procedure's cost. Additionally, in conventional methods, high-temperature long-time extractions are practiced which can accelerate the yields of hydrolysis products of 5-CQA (Pimentel-Moral et al., 2018). Therefore there is a requirement

to optimize a convenient method for the extraction of 5-CQA from hibiscus flowers using a minimum amount of solvent which is the least toxic in food applications, for example, ethanol. Additionally, the literature lacks evidence about the effect of the solvent mixture's pH on the extract's 5-CQA level. For instance, at higher pH values (5.0 to 9.0) CGA is easily hydrolyzed into products such as quinic acid, and caffeic acid, and with the effect of various conditions (e.g.; pH, and temperature), reversible isomerization can occur (Mok et al., 2022) which requires investigation in optimizing an efficient method.

Amongst various quantification methods of 5-CQA, high-performance liquid chromatography (HPLC) is the most dominating method even though it has several drawbacks. However, the extraction and analysis of 5-CQA in plant sources is challenging because of their diverse compositions and concentrations of active ingredients. For instance, in green coffee ethanolic extracts, interference of caffeine with 5-CQA at some wavelengths (274nm) can be observed in HPLC determinations. Additionally, the extraction solvents and method directly affect the determined 5-CQA levels in herbal infusions and there is a lack of information about HPLC methods optimized with each extraction method (Peng et al., 2005). Therefore, this study aimed to develop an efficient, convenient, and sustainable method for the extraction of 5-CQA from hibiscus flowers utilizing ethanol as the solvent and quantifying 5-CQA. Further, this study focused on evaluating the performance of two different analytical HPLC columns for the analysis of 5-CQA in ethanolic extracts of hibiscus flowers in terms of separation of 5-CQA, analysis time, and peak purity level. In this study, the optimization of the extraction method and 5-CQA quantification method was performed using Response Surface Methodology (RSM) utilizing a box-Behnken design which can investigate the effect of multiple independent variables on the dependent variable together.

2 Materials and Methods

2.1 Materials

Commercially dehydrated hibiscus (*Hibiscus sabdariffa* L.) flower samples used for the measurements were purchased from Natur Tea, Hungary. For extractions, Ultra-pure water was obtained from an ultra-pure water system (Milli Q-SQ 2 series, Germany), and absolute ethanol was purchased from VWR chemicals (France). The standard 5-CQA (purity ≥ 95%) was purchased from Merck Science Life Ltd. (Budapest, Hungary). For mobile phase preparation, acetonitrile of HPLC grade (Fisher Scientific, UK), and ortho-phosphoric acid of analytical grade (Lachner, Hungary) were used. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \times H_2O$), sodium acetate (CH_3COONa), acetic acid (CH_3COOH), and disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2H_2O$) were purchased from Merck, Germany.

2.2 Preparation of hibiscus extract

Hibiscus extracts were prepared in triplicates following a 3x3x3 factor-factorial design focusing on the effect of solvent composition, pH of the solvent mixture, and extraction time on the yield of 5-CQA extracted from the hibiscus flower. Treatment combinations described in Table 1 were selected after a preliminary study of existing literature. In all treatments, 1g of dehydrated hibiscus flowers was used to prepare 100 mL of extract. The pH adjustment was performed by using 10mL of respective buffer solution with 0.1M molarity; $NaH_2PO_4 \times H_2O$ with H_3PO_4 for the pH=2, CH_3COONa with CH_3COOH for the pH=4.5, and $NaH_2PO_4.H_2O$ with $Na_2HPO_4.2H_2O$ for the pH=7.0. All samples were maintained at the boiling temperature for the

respective extraction time mentioned in Table 1. After that, the extract was filtered into 1.5 mL vials using a syringe with a 0.22 µm hydrophilic filter for HPLC analysis.

Table 1 Factorial design (3^3) experimental values of tested independent variables for the extraction of 5-CQA from hibiscus flower

Treatment ID	Time (min)	Volume of ethanol (mL)	Volume of water (mL)	pH Value
T1	20	25	65	2.0
T2	20	25	65	4.5
T3	20	25	65	7.0
T4	20	50	40	2.0
T5	20	50	40	4.5
T6	20	50	40	7.0
T7	20	75	15	2.0
T8	20	75	15	4.5
T9	20	75	15	7.0
T10	40	25	65	2.0
T11	40	25	65	4.5
T12	40	25	65	7.0
T13	40	50	40	2.0
T14	40	50	40	4.5
T15	40	50	40	7.0
T16	40	75	15	2.0
T17	40	75	15	4.5
T18	40	75	15	7.0
T19	60	25	65	2.0
T20	60	25	65	4.5
T21	60	25	65	7.0
T22	60	50	40	2.0
T23	60	50	40	4.5
T24	60	50	40	7.0
T25	60	75	15	2.0
T26	60	75	15	4.5
T27	60	75	15	7.0

2.3 Preparation of 5-CQA standard solutions

A stock solution of 5-CQA at a concentration of 1mg/mL was prepared by dissolving 50.0mg of 5-CQA in 50 mL of ethanol. Working standard solutions of concentrations within the range of 5 to 50 µg/mL were prepared and injected into the HPLC system under determined conditions for developing a 5-CQA calibration curve by plotting concentration versus peak area. Separate 5-CQA calibration curves were developed for the two HPLC columns used in this study. Quantification of 5-CQA levels in each extract was performed using the calibration curves' regression equation of the best line of fit.

2.4 HPLC Analysis of 5-CQA in ethanolic hibiscus extracts (isocratic method)

The instrumental analysis of the 5-CQA was performed using an HPLC-PDA system (Jasco, Japan) equipped with a 4-line degasser (Jasco DG-2080-54), intelligent HPLC pump (Jasco PU-980), ternary gradient unit (Jasco LG-980-02), intelligent sampler (Jasco AS-2055), and a column thermostat (Phenomenex TS-130) maintained at 35˚C, and a column compartment. ChromeNAV software (Jasco, Japan) was used for data acquisition. Separation was achieved using two different columns (A: InterSustain Phenyl column with 5µm pore size, 3mm diameter, 150mm length, and B: Synergi Polar column with 4µm pore size, 4.6mm diameter, 250mm length) following an isocratic procedure. Columns were compared for the HPLC analysis of hibiscus extracts using a predetermined isocratic procedure based on preliminary studies. The mobile phase was composed of 7% acetonitrile, with 0.1% phosphoric acid, for column A, and 10% acetonitrile, with 0.1% phosphoric acid, for column B. Flow rate and injection volume in both columns were maintained as 1.2mL/min and 2µL respectively. The wavelength was set at 325 nm for monitoring the chromatographic profile. All measurements were done in triplicate.

2.5 Method Validation

Method validation was performed by calculating the calibration range, linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ) values for the calibration curves following the method explained by Chaowuttikul et al (2020). The specificity was analyzed by a peak purity test by comparing all the spectra within the chroatographic peak to the reference spectrum at the peak appex. The accuracy was tested by the recovery method. The percent recoveries based on each analytical column was calculated using a spiked test by injecting 100 µL of 1mg/mL 5-CQA standard solution into each sample (5 mL) in triplicates.

2.6 Experimental Design and Statistical Analysis

In this study, RSM occupying a box-Behnken design was used to assess the relationship between three independent variables (pH, ethanol concentration, and extraction time) which were coded into three levels (-1, 0, and +1), and the dependent variable (area of 5-CQA peak in chromatogram) and identify the optimal levels of the independent variables for the dependent variable. All analyses were conducted in triplicates. Minitab 19 software was used for the analysis of variance (ANOVA), response surfaces, regression equations, and designing the contour and surface plots. A confidence level of 95% was set as the basis for the determination of the significance of the difference. Validation of the RSM model was performed by analyzing the residual plots, model p-value, lack of fitness, determination coefficient (R^2), and $R^{2\ (adj)}$ values. Other data was analyzed using Microsoft Office Excel. All values were expressed as mean ± standard deviation (SD) values obtained in experiments performed in triplicate.

3 Results and Discussion

3.1 Optimization of HPLC conditions for the 5-CQA in hibiscus extracts

According to the literature, in HPLC, different chemicals such as formic acid, and phosphoric acid are used in the aqueous phase to improve the resolution, control ionization, and reduce peak tailing of compounds of interest (Chaowuttikul et al., 2020). In this study, after conducting preliminary trials with various mobile phases occupying numerous proportions of different aqueous phases and organic modifiers to separate 5-CQA in hibiscus, the most suitable mobile

phase that showed good resolution and symmetric peak shape was obtained using 7% acetonitrile and 0.1% phosphoric acid (column A) and 10% acetonitrile and 0.1% phosphoric acid (column B) with an isocratic program. The column temperature was held at 35˚C for the duration of the analysis to improve the retention time precision. In HPLC, the identification of compounds in herbal infusions depends on the retention time and UV-light spectral characteristics of the chromatographic peak of the standard solution. According to the literature, hydroxycinnamic acids have the maximum wavelength during 270 - 360 nm wavelengths (Yilmaz and Kolak, 2017). By comparing the UV spectra of standard 5-CQA at varying wavelengths, 325nm wavelength was identified as the optimum wavelength to detect 5-CQA.

3.2 Validation of the optimized method

To quantify the amount of 5-CQA in the herbal infusions of hibiscus, calibration curves for 5-CQA were prepared occupying each HPLC column used for the analysis in this study. The calibration curves for 5-CQA designed based on HPLC analysis with A and B columns are depicted in Figure 1. The regression equations for 5-CQA with A (y = 7364x - 3495) and B (y = 4859x - 3423) columns obtained in the form of y = ax + b demonstrated good linearity between concentration and peak area within the range of 5–50 µg/mL. The high correlation coefficient values (R_A = 0.99, R_B = 0.99) of 5-CQA denotes an excellent correlation between 5-CQA concentration and peak area. Similarly, the R^2 values were higher than 0.99 for both A and B columns, demonstrating the regression model fits the observed data satisfactorily. An analytical method is considered acceptable when the R^2 value is 0.99 or above (Chaowuttikul et al., 2020). Retention times for the standard 5-CQA in the HPLC analysis (at 325nm) using A, and B and columns were 5.69 and 12.1 min respectively. LOD and LOQ values for the 5-CQA with column A were 4.69µg/mL and 14.22µg/mL respectively. LOD and LOQ values for the 5-CQA with column B were 7.70µg/mL and 23.0µg/mL respectively. In quantification, it was confirmed that the spectral peaks for the hibiscus extracts were in line with the spectra of standard 5-CQA, which were further confirmed by spiked tests. Recovery percentages calculated with spiked samples were within the acceptable range of 80-120% (105.7% and 105.6% for columns A and B respectively). The percentage residual standard deviation (RSD %) values for the spiked samples were 2% and 3% for column A and B, respectively. RSD% values not more than 15% indicate the accuracy and repeatability of the tested RP-HPLC methods for the quantification of 5-CQA in hibiscus samples.

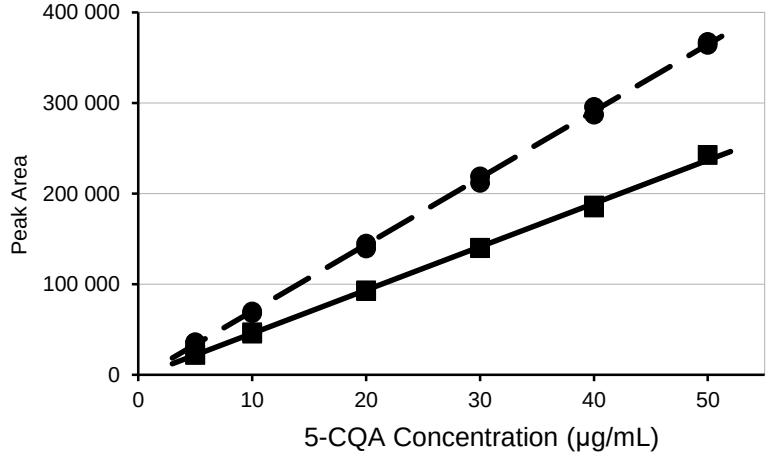

Figure 1 *Calibration curve for 5-CQA concentration developed for the two RP HPLC columns; InertSustain phenyl column depicted with a dashed line and round-shaped markers, Synergy Polar column depicted with a solid line with square-shaped markers*

According to the literature, when RP-HPLC is used for the separation of chlorogenic acids, the elution order of CGA isomers varies according to the provided conditions. For instance, the partition of an analyte between the column's packing material and eluent is affected by their polarities. In RP-HPLC, the polarity of the packing material is particularly lower than that of eluent. Hence, the analytes with higher polarity tend to distribute better in the eluent, eluting the analytes faster from the column. When referring to the structure, 5-CQA and 4-CQA have one axial and one equatorial hydroxyl residue making it less hydrophilic than 3-CQA which has two hydroxyl residues in an equatorial structure. Another main factor that determines the elution order and the degree of separation of the three CGA isomers under RP-HPLC is the functioning of the residual silanol group. Deineka et al (2019), revealed that when water–acetonitrile–formic acid or orthophosphoric acid is used as the mobile phase with an RP-HPLC column as the stationary phase with a low activity of silanol groups or polar functional, the order of CGA elution remains as 3CQA < 5QCA < 4CQA. Additionally, there is a substantial effect of the pH of the mobile phase on the retention of such acidic analytes (Jeon et al., 2017). However, the identification of 3-CQA and 4-CQA was not performed in this study due to the unavailability of standards, marking it as a limitation of this study. Figures 2 and 3 depict representative base-peak chromatograms of T2 hibiscus extract analyzed with the two HPLC columns. When the phenyl column was used for the analysis, at the occupied wavelength of 325nm, peak 2 was identified as 5-CQA by comparing with the standard 5-CQA spectra. However, when the polar column was used for the analysis, the elution of 5-CQA was recorded as the peak 3 (Figure 3). The extension of the retention times of 5-CQA in the polar column compared to the phenyl column can be due to the improved polar interactions offered by the polar column. Peak purity was calculated to determine the specificity of the method and confirmed that the purity levels of 5-CQA peaks were 81.28% and 79% in column A and B respectively. Peak purity test is important to find out whether the chromatographic peak of the analytes attributable with another compound. Chromatographic peaks which generate peak purity values of 100% confirm that no impurity was detected in the peak. However, the specificity reported in this study were comparatively low than the expected value demonstrating a possibility to contain some impurities. In contrast, the correlation coefficient values of the 5-CQA peak with 50µg/mL of 5-CQA standard were 0.9908 and 0.9965, in columns A and B, respectively, demonstrating both of these columns can be successfully used in the separation of 5-CQA in hibiscus extracts. Further, the comparison of 5-CQA yields from all studied hibiscus extracts as per the analysis with SynergiPolar and InertSustain Phenyl columns summarized that there is a determination coefficient of 0.9992 demonstrating the analytical values for the two studied RP-HPLC columns generate similar results (Figure 4).

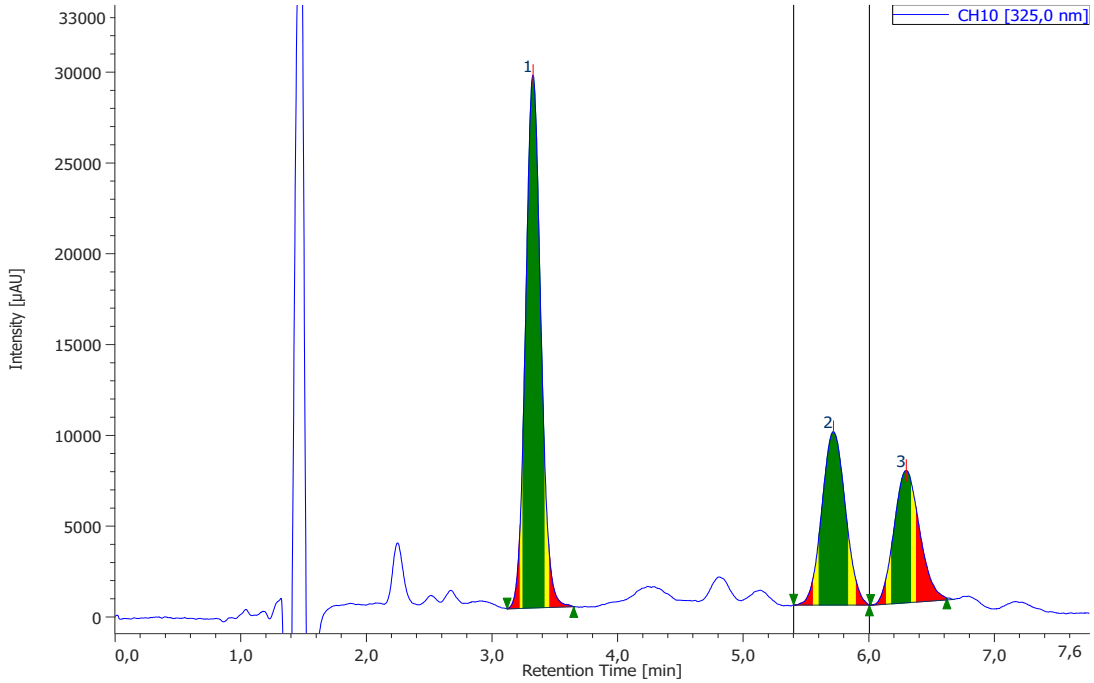

Figure 2 *Separation of chlorogenic acid with a mobile phase containing 7% acetonitrile and 0.1 % phosphoric acid with a flow rate of 1.2mL/min on InertSustain phenyl stationary phase at detector wavelength of 325 nm. Peak 2 was identified as 5-CQA. The purity of the 5-CQA peak was 81.28%. The green, yellow and red colours represent the high, medium and low purity at the peak's top position, respectively.*

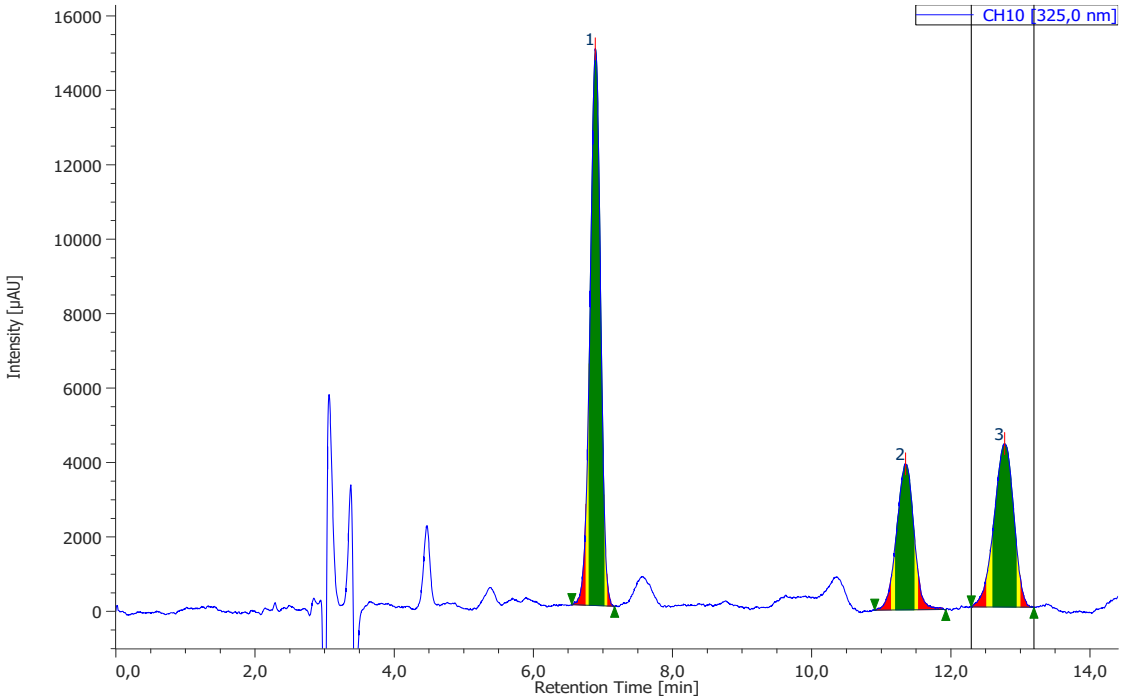

Figure 3 *Separation of chlorogenic acid with a mobile phase containing 10% acetonitrile and 0.1 % phosphoric acid with a flow rate of 1.2mL/min on SynergiPolar stationary phase at a detector wavelength of 325 nm. Peak 3 was identified as 5-CQA. The purity of the 5-CQA peak was 79%. The green, yellow and red colours represent the high, medium and low purity at the peak's top position, respectively.*

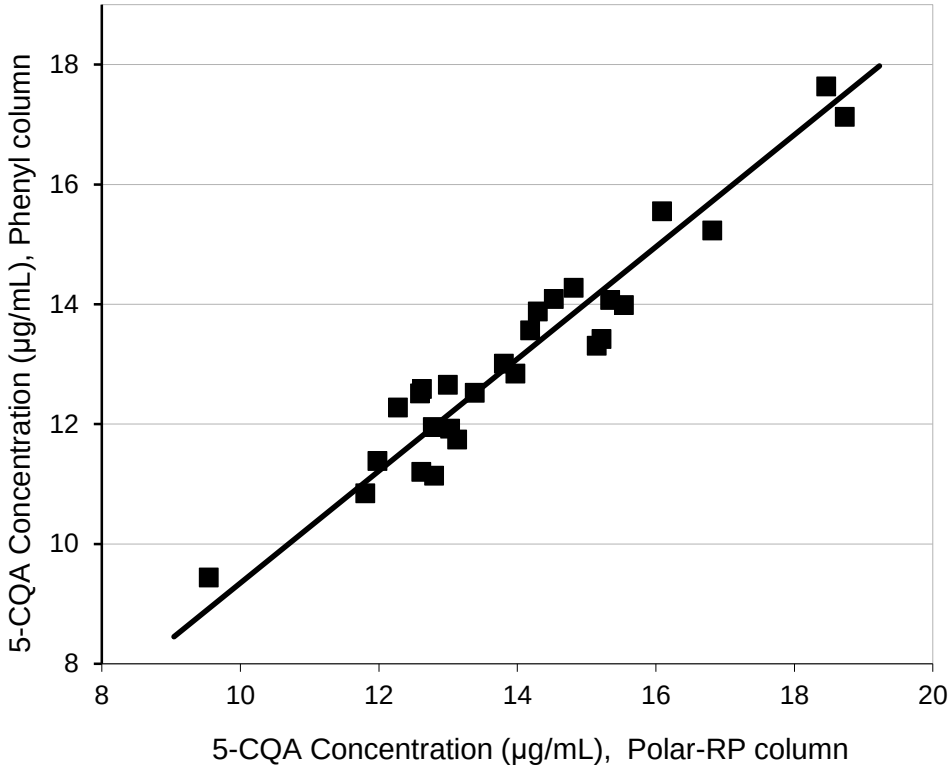

Figure 4 *Comparison of the 5-CQA concentration in hibiscus flower extracts analyzed with the two RP-HPLC columns; Phenylcolumn vs. Polar RP column*

3.3 Optimization of extraction conditions and quantification of extractable 5-CQA in hibiscus

The experimental results of 5-CQA detected in each hibiscus extract based on the HPLC analysis with two selected columns in this study are presented in Table 2. The 5-CQA levels were calculated using the equations generated according to the calibration curves. Accordingly, the 5-CQA concentration in 1mL of hibiscus extract ranged from 9.4 to 17.6 µg in HPLC analysis with the InertSustain column, while that was ranged from 9.5 to 18.5 µg in HPLC analysis with the SynergiPolar column. In both tested RP-HPLC columns, the highest 5-CQA yield (A=17.6µg/mL, B=18.5µg/mL) was reported in T2 extraction, which occupied 25% ethanol concentration, pH=4.5 buffer solution and 20min extraction time.

Table 2 The concentration of 5-CQA detected in Hibiscus flower extracts by HPLC analysis with InertSustain Phenyl Column and Synergi Polar Column

Treatment ID	InertSustain Phenyl Column	Synergi Polar Column
	Average 5-CQA concentration ± SD (µg/mL)	Average 5-CQA concentration ± SD (µg/mL)
T1	13.6±0.16	14.2±0.28
T2	17.6±0.24	18.5±0.18
T3	14.3±0.07	14.8±0.37
T4	14.1±0.23	14.5±0.44
T5	17.1±0.43	18.7±0.55
T6	13.3±0.63	15.1±0.30
T7	15.6±0.62	16.1±0.03
T8	11.2±0.23	12.6±0.20
T9	13.4±0.13	15.2±0.02
T10	11.4±0.25	12.0±0.20
T11	12.0±0.16	12.8±0.00
T12	13.9±0.09	14.3±0.18
T13	9.4±0.05	9.5±0.25
T14	11.7±0.24	13.1±0.15
T15	11.1±0.03	12.8±0.23
T16	14.1±0.18	15.3±0.12
T17	10.8±0.05	11.8±0.11
T18	14.0±0.08	15.5±0.48
T19	12.5±0.07	12.6±0.06
T20	12.5±0.13	13.4±0.45
T21	12.6±0.09	12.6±0.39
T22	12.8±0.07	14.0±0.15

T23	13.0±0.19	13.8±0.25
T24	15.2±0.30	16.8±0.02
T25	12.3±0.12	12.3±0.03
T26	11.9±0.09	13.0±0.26
T27	12.7±0.29	13.0±0.07

The effect of extraction time, solvent composition, and pH value on the peak area of the 5-CQA chromatogram was extensively investigated in this study. The residual plots (Figure 5) for the analysis of peak area based on the two analytical columns used in this study demonstrate that the data is randomly distributed being independent of each other following a normal distribution. Additionally, there is an equality of variance in the analyzed data for both columns. According to the multivariate regression analysis conducted occupying the RSM model, the R^2 was 62.34% while $R^{2(adj)}$ was 45% when the InertSustain column was used. The R^2 was 56.78% while $R^{2(adj)}$ was 37% when the SynergyPolar column was used. These R^2 and $R^{2(adj)}$ values indicate that the RSM model moderately fits with the analyzed data due to the significance of some factors considered in this study. The model p-values for columns A and B were 0.007 and 0.022 while the lack of fit values for both columns was 0.2 showing that this model can be used for further studies. However, it is suggested to improve the existing model further by considering cubic terms and including other random variables that affect the outcome as well.

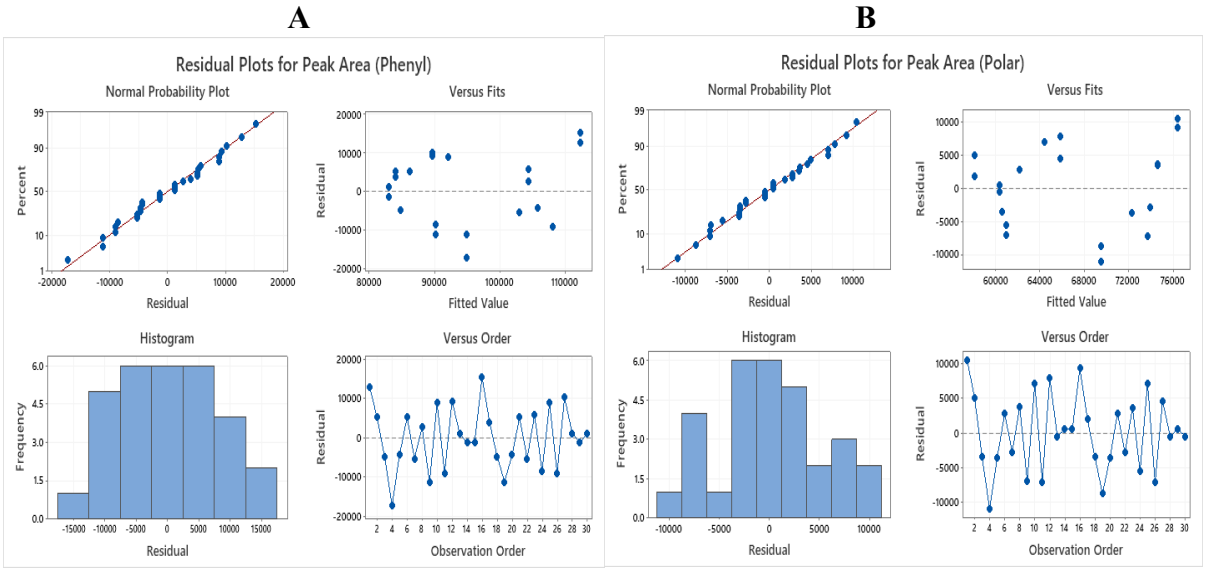

Figure 5 *Residual plots for the peak area of (A) InertSustain Phenyl column and (B) SynergiPolar column according to the response surface model*

For the area of the 5-CQA peak with column A, there was no significant effect from the linear variables ($p>0.05$), while time with ethanol concentration (C_{ETOH}) was the only significant two-way interaction ($p<0.05$) between the factors. However, in quadratic terms, there was a significant interaction with the square effect of time and pH ($p<0.05$). For the area of the 5-CQA peak with column B, pH was the only significant linear variable ($p<0.05$) while time with C_{ETOH} was the only significant two-way interaction ($p<0.05$) between the factors. Regarding the quadratic terms also, there was a significant interaction with the square effect of time and pH ($p<0.05$) (Table 3). Regression equations (Eq. 1, 2) designed based on the RSM models

depicted below describe the relationship between the independent and dependent variables. According to both equations, pH had a positive effect indicating that the increase in pH favored 5-CQA extraction while extraction time had a negative effect indicating that the increase in extraction time was not desired for the 5-CQA extraction. However, the effect of ethanol concentration on the dependent variable was negative according to column A and positive according to column B.

Table 3 ANOVA for polynomial surface response model of all variables depending on the two HPLC columns used for the analysis

Factors	P-value	P-value
	InertSustain Phenyl	SynergyPolar
Linear effect		
Time	0.073	0.175
C_{ETOH}	0.098	0.513
pH	0.121	0.047*
Quadratic effect		
Time*Time	0.035*	0.049*
C_{ETOH}*C_{ETOH}	0.384	0.796
pH*pH	0.019*	0.046*
Interaction effect		
Time*C_{ETOH}	0.010*	0.009*
Time*pH	0.137	0.261
C_{ETOH}*pH	0.146	0.244

**Significant at $p<0.05$*

$$Peak\ Area_{(InertSustain\ Phenyl)} = 153249 - 3153\ Time - 41\ C_{ETOH} + 1134\ pH + 29.15\ Time*Time + 0.01\ C_{ETOH}*C_{ETOH} - 3\ pH*pH + 5.13\ Time*C_{ETOH} + 57.6\ Time*pH - 53.8\ C_{ETOH}*pH \quad (1)$$

$$Peak\ Area_{(Synergy\ Polar)} = 90261 - 1945\ Time + 276\ C_{ETOH} + 2797\ pH + 18.12\ Time*Time - 2.98\ C_{ETOH}*C_{ETOH} - 205\ pH*pH + 2.62\ Time*C_{ETOH} + 26.1\ Time*pH - 18.9\ C_{ETOH}*pH \quad (2)$$

The contour plots and surface response plots of the peak area of 5-CQA with the interaction effect of C_{ETOH} with extraction time, pH with extraction time, and C_{ETOH} with pH, as per the HPLC analysis with columns A and B are depicted in Figure 6 and 7 respectively. It is a common fact that the chemical composition of hibiscus extracts obtained by different extraction methods can be different from each other depending on factors such as the type and concentration of solvent used, extraction temperature, time, and technology. Additionally, the HPLC conditions affect the separation of each bioactive compound. According to the extraction trends depicted in Figures 6 and 7, with extended extraction times, pH levels above 6 yield higher concentrations of 5-CQA than with lower pH levels, without being affected by the HPLC column used for the analysis. Similarly, according to the analysis with both columns, higher pH values generated higher yields of 5-CQA up to the 60% concentration of ethanol used for the extraction. This can be due to the reversible isomerization of hydrolyzed products of 5-CQA, which mainly involves acyl groups, at higher pH values with the effect of various conditions such as temperature, and solvent concentration (Mok et al., 2022). Moreover, with all tested ethanol concentrations and pH levels, lower extraction times yielded higher 5-CQA concentrations than extended heat treatments, when analyzed with both columns A and B.

It was determined from the RSM study that 25% ethanol, pH=7 buffer solution, and heat treatment for 25 minutes as the optimum parameters for maximizing the 5-CQA yield with a

composite desirability value of 0.9455 and 0.9498 for columns A and B, respectively. The experimental values for 5-CQA yield under the optimal conditions were 1.43mg and 1.48mg from 1g of dry hibiscus flower based on the analysis with columns A and B respectively. According to Mok et al (2019), a maximum of 0.12 mg of CGA was extracted from 1g of dry hibiscus flower using 50% methanol for 6 hrs. Accordingly, the present study reveals that the use of ethanol as the solvent for the extraction of 5-CQA is more effective than methanol. This can be further explained by the fact that the methods that can reduce the polarity of water by cleaving hydrogen ions in water can increase the solubility of 5-CQA in water because 5-CQA is a substance with low polarity. Ethanol is less polar than methanol due to the presence of a larger alkyl group in ethanol. Hence a solvent mixture with low polarity becomes ideal for the extraction of 5-CQA (Mok et al., 2022). Previous studies have also observed that the heat treatment significantly affected the extraction of 5-CQA. More particularly, 5-CQA has an acyl group bound to the hydroxyl group on carbon 5 of quinic acid which makes it more sensitive to heat treatments. Accordingly, with temperatures above 110˚C combined with longer extraction times, the yield of 5-CQA was decreased (Mok et al., 2022). However, the method presented in the current study becomes efficient and sustainable in terms of solvent usage and energy consumption for the heat treatment applied in 5-CQA extraction.

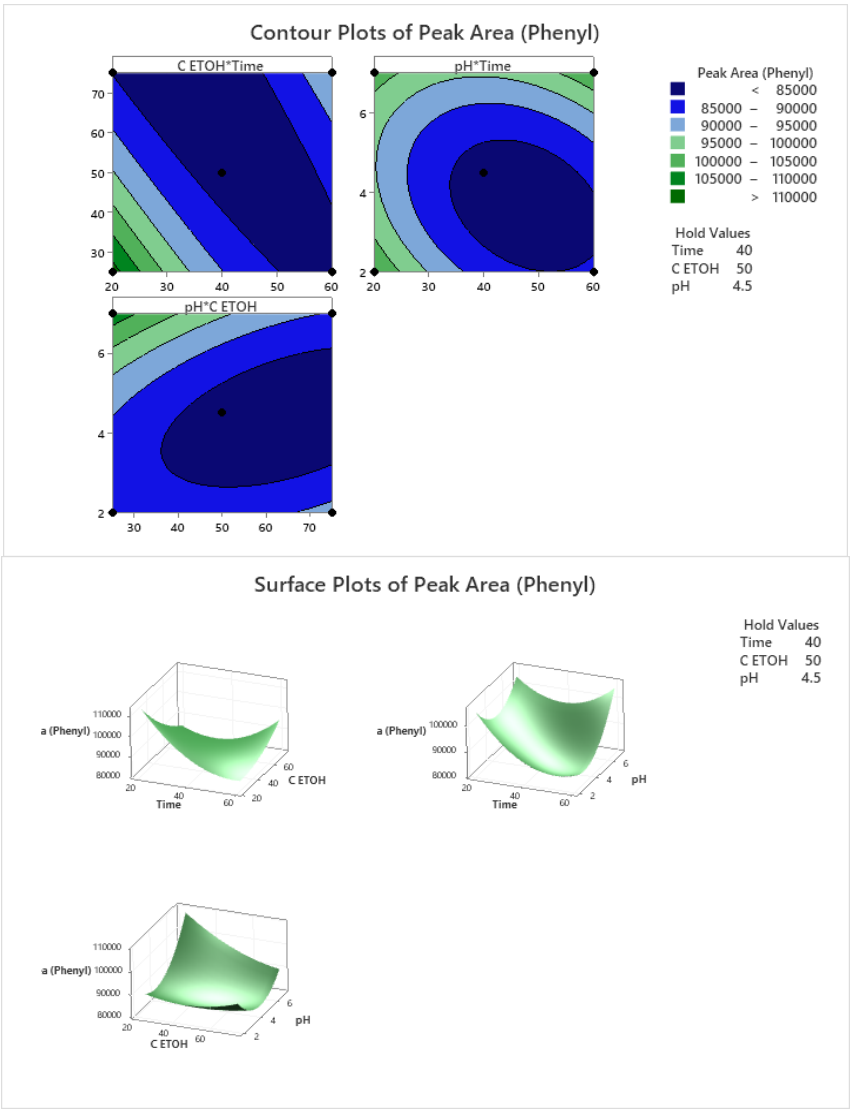

Figure 6. *Contour plots and surface plots of peak area of 5-CQA with the interaction effect of C_{ETOH} with extraction time, pH with extraction time, and C_{ETOH} with pH, as per the HPLC analysis with InertSustain phenyl column*

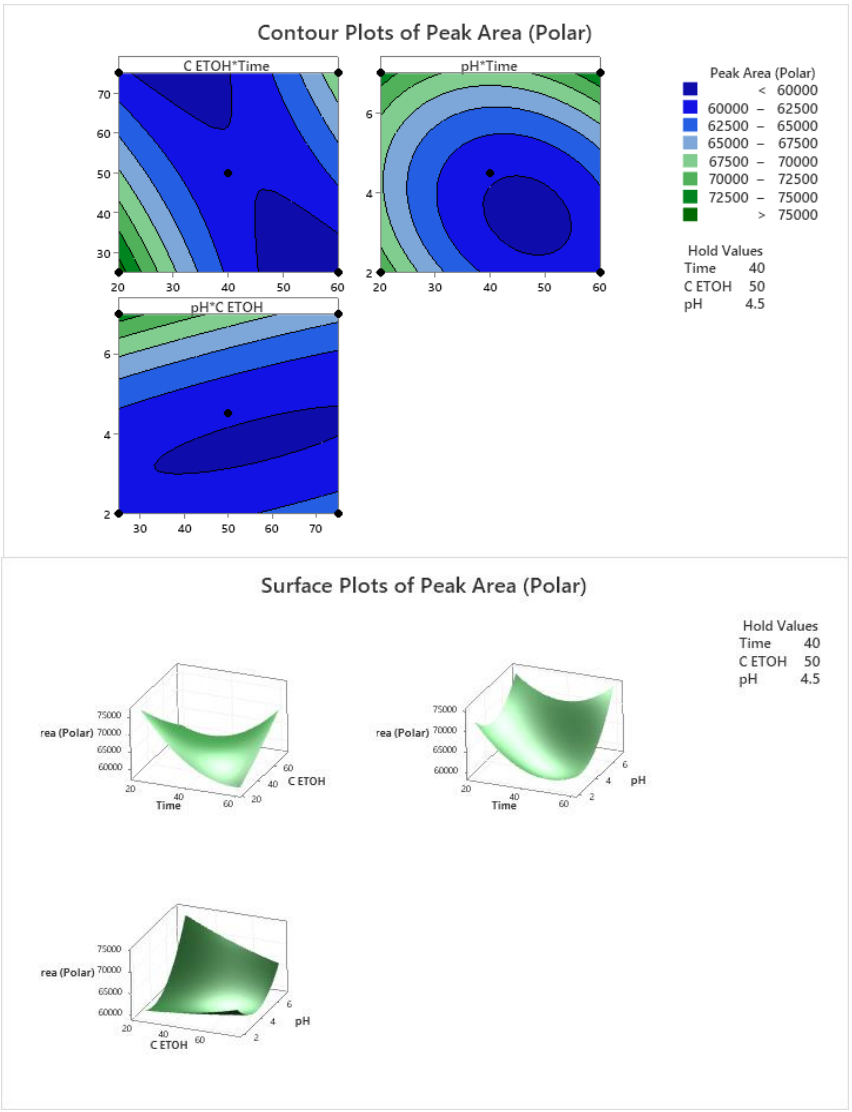

Figure 7. *Contour plots and surface plots of peak area of 5-CQA with the interaction effect of C_{ETOH} with extraction time, pH with extraction time, and C_{ETOH} with pH, as per the HPLC analysis with SynergyPolar column*

5. Conclusions

This study suggested that the optimum extraction conditions for maximum yield of 5-CQA from hibiscus flower are 25% ethanol, pH=7, and 20 minutes heat treatment concerning the HPLC analysis with both InertSustain phenyl and Synergi Polar columns. The experimental values for 5-CQA yield under the optimal conditions were 1.43mg and 1.48 mg from 1g of dry hibiscus flower, analyzed with InertSustain phenyl and Synergi Polar columns respectively. This extraction method can be justified from ecological and economic perspectives since it uses minimum amounts of ethanol, in addition to the short-period heat treatments, which can be effectively used in food manufacturing and pharmaceutical applications. The study revealed that the RSM model designed in this study can be used for the predictions in future studies, however, further improvements in the existing model by considering cubic terms and including other random variables that affect the outcome are suggested.

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