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Comparison of extraction methods optimised by RSM for extraction of some bioactives from liquorice samples

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Abstract: This study compared the extraction efficiency of 3 different techniques, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and the classical extraction method, in obtaining some bioactive and allergen compounds, namely liquiritin (LQ), glycyrrhizin (GL), glycyrrhetinic acid (GA), and carbenoxolone (CBX), from liquorice samples of *Glycyrrhiza glabra* grown in Turkey. Extraction parameters for each technique were optimised by the response surface methodology–central composite design approach. Concentrations of LQ, GL, GA, and CBX, which were selected as the responses of selected independent variables, were determined by liquid chromatography–electrospray ionisation tandem mass spectrometry. One-way variance analysis was performed to evaluate the significance of the results. MAE was revealed as the most effective technique for extracting LQ (mean: 8.52 mg g^{-1}), GL (mean: 76.65 mg g^{-1}), and GA (mean: 1138.84 µg g^{-1}) from liquorice, while the classical technique was the most effective for obtaining CBX (mean: 5.75 µg g^{-1}).

Key words: LC–ESI–MS/MS, liquorice, microwave, response surface methodology ultrasound

1. Introduction

Liquorice (*Glycyrrhiza*) species belong to the legume family (Fabaceae) and are grown in Europe, Asia, North and South America, and Australia. The genus *Glycyrrhiza* includes about 30 species worldwide (Lange, 1998; Isbrucker and Burdock, 2006; Bao and Larsen, 2010). In Turkey, the largest producer and exporter of *Glycyrrhiza glabra*, 5 species can be found, 2 of which have 2 subspecies: *G. asymmetrica* (endemic), *G. iconica* (endemic), *G. glabra*, *G. flavescens* subsp. *flavescens*, *G. flavescens* subsp. *antalyensis* (endemic), *G. echinata* subsp. *macedonica*, and *G. echinata* subsp. *echinata* (Çetin et al., 2015).

Liquorice species are often used for their antioxidant, demulcent, expectorant, antidepressant, antitussive, laxative, antiinflammatory, antiviral, antiallergic, and gastroprotective properties in herbal medicine (Tao et al., 2013). Moreover, they have anticarcinogenic effects (Shabkhiz et al., 2016). Large amounts of liquorice and its extracts are used by the food, tobacco, and pharmaceutical industries (Tao et al., 2013) as they provide sweetness, flavour enhancement, and foaming properties to various products such as cough syrups, baked goods, chewing gum, tobacco, and beverages (Hough et al., 1973; Kitagawa, 2002; Mukhopadhyay and Panja, 2008).

Liquorice contains bioactive compounds such as the major saponin glycyrrhizin (GL), its sapogenin glycyrrhetinic acid (GA), the phenolic compound liquiritin (LQ), and carbenoxolone (CBX), an analogue of GA. Among these phytochemicals, triterpene saponins are used as sweeteners because of their higher solubility and enduring sweetness properties (Liu et al., 2000); they are also formulated into a botanical dietary supplement for detoxification (Chin et al., 2007). In recent years, studies have indicated that liquorice saponins have various hepatoprotective, anticancer, antiviral, antiinflammatory, and neuroprotective effects (Cherng et al., 2006; Li et al., 2010; Zheng et al., 2010; Ashfaq et al., 2011; Gao and Zheng et al., 2011).

Bioactive compounds can be extracted with conventional technologies such as Soxhlet extractors or by classical extraction, but these have the disadvantages of conventional systems such as high extraction time, increased consumption of energy and harmful chemicals, low extraction efficiency, and also possible degradation of bioactives. These shortcomings have resulted in the introduction of new "green" technique separation methods such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), which typically use

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less solvent and energy (Kaderides et al., 2019). Microwaves are nonionizing electromagnetic waves with a frequency between 300 MHz and 300 GHz. They instantly heat polar molecules such as H_2O inside the plant cell via an evaporation process, which causes huge pressure in plant cell walls. This pressure stretches and destroys the cell wall, thus facilitating the penetration of bioactive compounds from the broken cells and improving the extraction yield (Mandal et al., 2007). On the other hand, UAE uses high-frequency sound waves (20–50 kHz) to destroy the plant cell wall and leads to the isolation of phytochemicals from plant materials due to its high-strength mechanical and thermal effects. Although these 2 extraction techniques have their advantages, such as shorter extraction time and lower solvent consumption, MAE causes inhomogeneous heating, while the thermal effect of UAE is relatively weak (Wang et al, 2018).

Many techniques have been used to extract bioactive compounds from liquorice samples. For instance, Liao et al. (2016) isolated GL from *G. uralensis* by using an ultrasonic bath system. Shabkhiz et al. (2016) found that superheated water extraction had a relatively higher efficiency than a Soxhlet extractor and UAE for obtaining GL from liquorice roots and, like Hedayati and Ghoreishi (2015), used the response surface methodology (RSM) strategy to optimise extraction conditions. RSM is based on the analysis of response as influenced by several factors, and its goal is to find the optimal condition of the response (Bezerra et al., 2008). Baite and Das (2018) optimised the extraction of GL from liquorice using a single screw extruder. Yang et al. (2013) proposed an ionic liquid-based, ultrasonic-assisted method for extracting isoliquiritigenin, LQ, and GL from liquorice and optimised the technical parameters, including soaking time, solid-to-liquid ratio, ultrasonic power, and duration. However, no other studies have been published on the optimisation of the extraction parameters (to extract GL, GA, LQ, and CBX from *G.* glabra) by 3 different extraction methods and the comparison of the efficiency of these extraction methods.

The current study aims to demonstrate the effects of 3 different extraction methods, namely UAE, MAE, and classical extraction, on some bioactive and allergen compounds (GL, GA, LQ, and CBX) from liquorice samples of *G. glabra* grown in Turkey. Furthermore, technical parameters such as the temperature, time, solvent-to-raw material ratio, amplitude (%), and microwave power were optimised using RSM.

2. Material and methods

2.1. Raw material and chemicals

Glycyrrhiza glabra L. was purchased from a national beverage industry in Hatay, Turkey. Acetonitrile and methanol were supplied by Merck (Germany). The distilled water for preparing all required solutions was from a Milli-Q ultrapure water purification system (Millipore, Molsheim, France). Standards of GL, GA, CBX, and LQ were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.1. Preparation of standards

The GL, GA, CBX, and LQ standards were weighed in a 10-mL volumetric flask and dissolved with methanol to prepare stock solutions. The stock solutions were subsequently diluted with 1:1 (v/v) methanol:water to provide a range of working solutions.

2.2. Preparation of liquorice extracts

Liquorice roots were cleaned to remove soil residues and ground in a high-speed blender until pulverised into a free-flowing powder. The resultant powders were extracted with 1:1 (v/v) methanol:water according to the experimental design generated by the RSM–central composite design (CCD) for each extraction method. The final extracts were filtered, diluted by 10,000 times (for GL, GA, and LQ), and injected into a liquid chromatography–electrospray ionisation tandem mass spectrometry instrument (LC–ESI–MS/MS) (see Section 2.4) for quantification of GL, GA, and LQ. The undiluted extracts were also injected into the system to determine the CBX content.

2.3. Optimisation of extraction methods

The operating parameters for each method were optimised by RSM–CCD using Design-Expert 7.0.0. software (Stat-Ease, Minneapolis, MS, USA). Contents of GL, GA, LQ, and CBX were chosen as the responses of selected independent variables, which were given for each extraction method.

2.3.1. Microwave-assisted extraction

MAE was carried out in a microwave digestion system (Milestone Start D, Sorisole, Bergamo, Italy). Extraction time, the ratio of solvent to raw material, and microwave power were selected as independent variables for method optimisation. The GL, GA, LQ, and CBX values of the liquorice (*G. glabra*) obtained according to RSM–CCD are shown in Table 1.

2.3.2. Ultrasound extraction using an ultrasonic processor

An ultrasonic processor (Cole–Parmer, Vernon Hills, IL, USA) was used in the present study. Amplitude, extraction time, and the ratio of solvent to raw material were selected as independent variables for optimisation of the technique. The values of GL, GA, LQ, and CBX corresponding to the independent variables are shown in Table 2.

2.3.3. Classical extraction

Classical extraction was performed in bath water (USC300TH, VWR). Temperature, time, and the solvent-to-raw material ratio were selected as the independent variables for optimisation of the classical extraction method. The values of GL, GA, CBX, and LQ obtained by classical extraction for the experimental conditions are given in Table 3.

Table 1. Microwave-assisted extraction central composite design with the experimental values of the responses.

Run	Factor A, solvent:sample (mL/g)	Factor B, power (W)	Factor C, time (min)	Response 1, GL (Y1) (mg/g)	Response 2, GA (Y2) (μg/g)	Response 3, CBX (Y3) (μg/g)	Response 4, LQ (Y4) (mg/g)
1	13.2	325.0	4.5	76.1	604.7	0.9	7.5
2	30.0	535.2	4.5	120.4	787.9	2.7	7.3
3	30.0	114.8	4.5	59.7	1287.5	4.5	12.0
4	20.0	450.0	7.0	94.3	812.6	1.2	3.9
5	20.0	450.0	2.0	84.6	802.4	1.4	5.4
6	30.0	325.0	4.5	76.5	1145.9	2.7	8.3
7	20.0	200.0	2.0	68.0	1096.6	4.1	15.3
8	40.0	450.0	2.0	72.5	1239.9	4.0	4.4
9	46.8	325.0	4.5	52.0	1783.4	4.9	9.6
10	20.0	200.0	7.0	79.4	1231.0	2.1	13.0
11	30.0	325.0	0.3	73.2	1164.4	3.4	6.3
12	40.0	450.0	7.0	95.9	1548.4	3.7	5.6
13	30.0	325.0	4.5	76.7	1156.6	2.5	8.3
14	30.0	325.0	4.5	76.3	1155.4	2.5	8.3
15	40.0	200.0	2.0	62.5	1290.6	4.0	12.0
16	30.0	325.0	4.5	75.9	1156.5	2.5	8.4
17	30.0	325.0	8.7	79.3	1169.6	1.3	8.7
18	30.0	325.0	4.5	76.2	1156.3	2.5	8.3
19	40.0	200.0	7.0	57.5	1030.4	3.3	9.2
20	30.0	325.0	4.5	76.0	1156.9	2.5	8.4

2.4. Instrument and conditions

Quantitative determination of GL, GA, CBX, and LQ was performed using LC–ESI–MS/MS (Zivak Tandem Gold, İstanbul, Turkey) in negative-ion mode. Chromatographic separation of the compounds was achieved on a Symmetry C18 column (2.1 mm × 150 mm I.D., particle size 5 μm; Waters, Milford, MA, USA). The mobile phase consisted of deionised water (A) and methanol (B). The analysis was carried out at a constant flow (300 μL min^{-1} under a gradient program using the following steps; 0:00–2:30 min, 10% B; 2:30–4:00 min, 50% B; 4:00–10:00 min, 90% B; 10:00–12:00 min, 90% B; 12:00–15:00 min, 50% B; and 15:00–20:00 min, 10% B. The instrument conditions were as follows: 5000 V ion spray voltage; 35 psi nebuliser gas (nitrogen); 50 °C source temperature; and 350 °C drying gas temperature. Mass parameters were optimised using standard solutions of GL, GA, CBX, and LQ. All of the transition ions and parameters, which were determined after optimisation using standard solutions of each compound, are listed in Table 4.

2.5. Statistical analysis

The significance of the differences of target compounds was calculated by one-way analysis of variance (ANOVA) ($P < 0.05$) using IBM SPSS Statistics 23 (IBM Corp., Armonk, NY, USA) to decide the most efficient extraction technique among those evaluated. RSM was applied for optimisation of the technical parameters using Design-Expert 7.0.0. software. The 3D graphs of bioactives optimised by RSM are shown in Figures 1–10.

3. Results and discussion

3.1. Optimisation of extraction methods

3.1.1. Microwave-assisted extraction

According to the ANOVA table of results obtained for GL, the model F-value of 4306.52 implied that the model was significant. The significant model terms were A, B, C, AB, AC, BC, A^2, B^2, ABC, A^2B, A^2C, and AB^2. The lack-of-fit F-value of 0.02 implied that the lack of fit was nonsignificant. A nonsignificant result for this test indicates that the model is a good fit for the data, and this applies to all of the results. While the predicted R-squared (pred-R^2) was 0.9998, the adjusted R-squared (adj-R^2) was 0.9997. The final equation for GL is shown below:

$$Y1 = +76.26 - 7.18A + 18.02B + 1.81C + 2.13AB - 0.36AC + 3.34BC - 4.32A^2 + 4.88B^2 + 6.711E - 003C^2 + 3.75ABC - 8.04A^2B + 3.12A^2C + 2.44AB^2 \quad (1)$$

Table 2. Ultrasound extraction using an ultrasonic processor central composite design with the experimental values of the responses.

Run	Factor A, solvent:sample (mL/g)	Factor B, amplitude (%)	Factor C, time (min)	Response 1, GL (Y1) (mg/g)	Response 2, GA (Y2) (μg/g)	Response 3, CBX (Y3) (μg/g)	Response 4, LQ (Y4) (mg/g)
1	30.0	55.0	20.0	43.4	3.4	0.4	1.6
2	13.2	55.0	20.0	35.8	6.0	0.2	1.2
3	46.8	55.0	20.0	42.7	7.9	0.6	1.7
4	20.0	80.0	30.0	31.3	2.6	0.3	1.9
5	30.0	55.0	20.0	41.1	3.4	0.4	2.1
6	30.0	55.0	20.0	43.8	3.3	0.5	1.7
7	40.0	80.0	10.0	36.6	9.0	0.5	0.7
8	30.0	55.0	20.0	41.4	3.6	0.3	1.6
9	20.0	30.0	30.0	27.7	2.2	0.3	0.9
10	20.0	80.0	10.0	27.9	1.7	0.3	0.7
11	30.0	55.0	3.2	32.2	3.0	0.7	0.5
12	40.0	30.0	30.0	39.3	2.3	0.8	0.6
13	40.0	80.0	30.0	47.0	3.4	0.9	0.5
14	30.0	55.0	36.8	40.6	4.1	0.6	0.9
15	30.0	97.0	20.0	49.3	2.0	0.5	0.8
16	20.0	30.0	10.0	35.1	8.0	0.7	0.9
17	30.0	20.0	20.0	27.5	5.6	1.0	0.5
18	40.0	30.0	10.0	54.2	10.3	1.3	0.9
19	30.0	55.0	20.0	41.8	3.8	0.3	1.6
20	30.0	55.0	20.0	43.0	3.6	0.4	1.7

The ANOVA table of the GA results showed that the model was significant (model F-value of 6536.46). The model terms A, B, AB, AC, BC, A^2, B^2, C^2, ABC, A^2B, A^2C, and AB^2 were significant. Assessment of the lack of fit (F-value of 0.00) indicated that the model was a good fit. Pred-R^2 was 0.9999 and adj-R^2 was 0.9998. The final equation for GA is provided below:

$$Y2 = +1154.60 + 350.44A - 148.52B + 1.55C + 147.48AB - 12.03AC + 55.57BC + 13.93A^2 - 41.36B^2 + 4.35C^2 + 86.62ABC + 117.84A^2B + 22.56A^2C - 204.59AB^2 \quad (2)$$

According to the ANOVA table of the CBX results, the model F-value of 876.20 showed that the model was significant. All of the model terms were significant. The lack-of-fit F-value of 0.06 was nonsignificant. Pred-R^2 was 0.9978 and adj-R^2 was 0.9983. The final equation for CBX is given below:

$$Y3 = +2.56 + 1.20A - 0.52B - 0.62C + 0.50AB + 0.14AC + 0.28BC + 0.12A^2 + 0.37B^2 - 0.064C^2 - 0.17ABC + 0.15A^2B + 0.22A^2C - 0.42AB^2 \quad (3)$$

For LQ, the ANOVA table indicated a model F-value of 9105.43, which implied that the model was significant. All of the model terms were significant. The lack-of-fit F-value of 0.14 was nonsignificant. Pred-R^2 was 0.9996 and adj-R^2 was 0.9998. The final equation for LQ is indicated below:

$$Y4 = +8.35 + 0.62A - 1.41B + 0.70C + 0.98AB + 0.27AC + 0.61BC + 0.079A^2 + 0.46B^2 - 0.29C^2 + 0.41ABC - 2.36A^2B - 1.38A^2C - 1.39AB^2 \quad (4)$$

The conditions for point prediction given by the RSM–CCD were 4.50 min of microwave extraction at 325 W, and the ratio of solvent-to-raw material was 30.00. Under these conditions of point prediction, the GL, GA, CBX, and LQ values of *G. glabra* reached 76.25 mg g^{-1}, 1154.6 μg g^{-1}, 2.55 μg g^{-1}, and 8.35 mg g^{-1}, respectively. According to the desirability function method, the optimum extraction conditions were 10.09 solvent-to-raw material ratio, 100.31 W, and 7.00 min for microwave extraction. The desirability value of the optimum extraction was 100%. At this value, the amount of GL, GA, CBX, and LQ recovered was 128.33 mg g^{-1}, 1979.36 μg g^{-1}, 4.92 μg g^{-1}, and 35.44 mg g^{-1}, respectively. The yield of GL increased with increasing microwave power, while the yield of GA and LQ decreased. In addition, an increase of GL and LQ yield and a decrease of CBX yield were observed with an increase in extraction time. Furthermore, with an increase in the solvent/sample

Table 3. Classical extraction central composite design with the experimental values of the responses.

Run	Factor A, solvent:sample (mL/g)	Factor B, temperature (°C)	Factor C, time (min)	Response 1, GL (Y1) (mg/g)	Response 2, GA (Y2) (µg/g)	Response 3, CBX (Y3) (µg/g)	Response 4, LQ (Y4) (mg/g)
1	40.0	55.0	60.0	33.6	80.8	5.2	5.4
2	40.0	55.0	60.0	32.7	81.0	5.5	4.5
3	40.0	55.0	60.0	33.0	81.2	5.3	5.2
4	40.0	55.0	9.6	60.0	35.5	5.0	7.8
5	30.0	70.0	30.0	56.1	43.3	4.7	6.6
6	40.0	55.0	60.0	32.9	81.2	5.7	5.0
7	50.0	40.0	90.0	27.4	58.7	8.0	4.3
8	56.8	55.0	60.0	35.3	53.7	7.7	6.1
9	40.0	29.8	60.0	22.2	39.5	7.8	2.4
10	50.0	70.0	90.0	18.6	40.6	6.9	14.1
11	30.0	70.0	90.0	52.9	29.2	3.8	6.2
12	50.0	70.0	30.0	48.6	34.3	7.1	4.2
13	40.0	55.0	60.0	32.3	81.6	5.4	5.3
14	40.0	80.2	60.0	45.9	21.2	4.8	4.6
15	40.0	55.0	110.5	27.2	69.5	7.9	8.6
16	40.0	55.0	60.0	32.3	81.8	5.5	5.4
17	30.0	40.0	30.0	28.8	20.3	4.6	5.8
18	23.2	55.0	60.0	27.2	8.7	2.3	7.9
19	50.0	40.0	30.0	30.5	29.8	8.5	3.8
20	30.0	40.0	90.0	25.4	25.3	3.3	6.1

Table 4. Precursor-product ions and parameters for MRM of compounds used in present study.

Analytes	Precursor ion	Screening ion	Confirmation ion	Capillary voltage	Collision energy
	(m/z)	(m/z)	(m/z)	(V)	(eV)
Glycyrrhizin	822.1	351	193	80	50
Glycyrrhetinic acid	470	425	355	60	40
Liquiritin	417.5	254.9	135.1	60	20
Carbenoxolone	570.1	469.7	100.2	80	30

ratio, an increase in yields of GA, CBX, and LQ was observed. The extraction solvent could efficiently absorb microwave energy and lead to efficient heating due to the solvents' polar properties (Yan et al., 2010). The increase in microwave power can boost the penetration of solvent into the plant material and efficiently transfer bioactives via molecular interaction with the electromagnetic field. Moreover, it allows energy transfer to the solvent and sample (Guo et al., 2019). Due to the fact that microwave irradiation accelerates cell rupture by suddenly increasing the temperature and increasing internal pressure inside the cells of a plant sample, the surface of the sample is significantly destroyed. While rupturing the plant cells, irradiation promotes rapid penetration of the bioactives within the cells into the surrounding solvents (Zhang et al., 2008) and increases extraction efficiency. On the other hand, higher microwave power may lead to thermal degradation, polymerization, and oxidation of the bioactive compounds and thus lead to a decrease in yield (Mandal and Mandal, 2010; Guo et al., 2019).

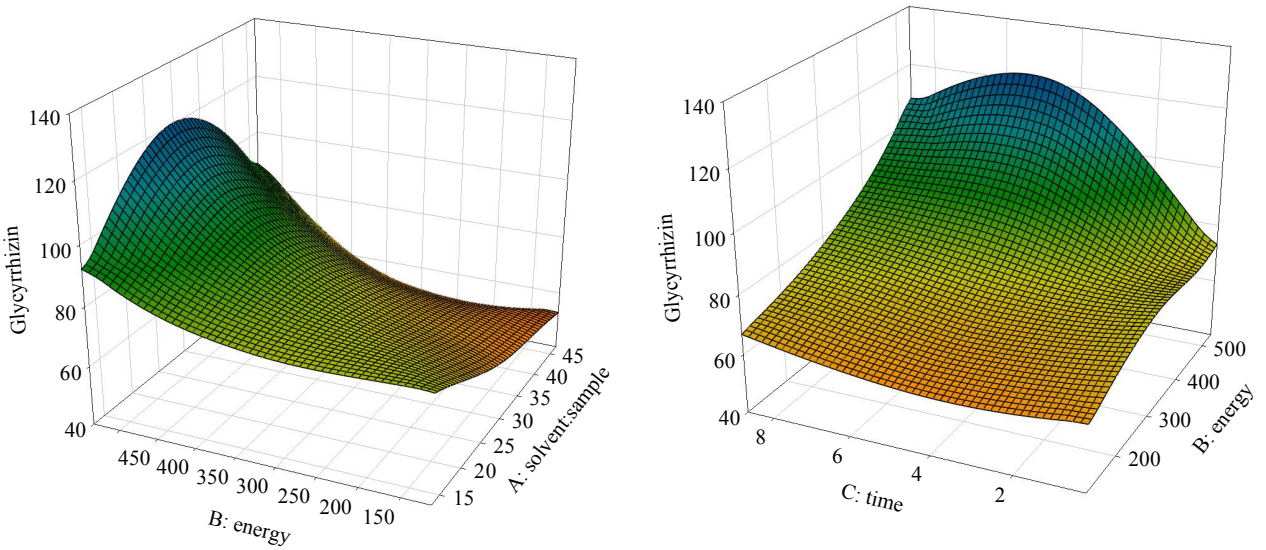


Figure 1. Response surface plots showing the effects of variables on value of GL: interaction of energy (B) and solvent:sample ratio (A); interaction of time (C) and energy (B) obtained by microwave-assisted extraction. Results were obtained based on 3 replicates.

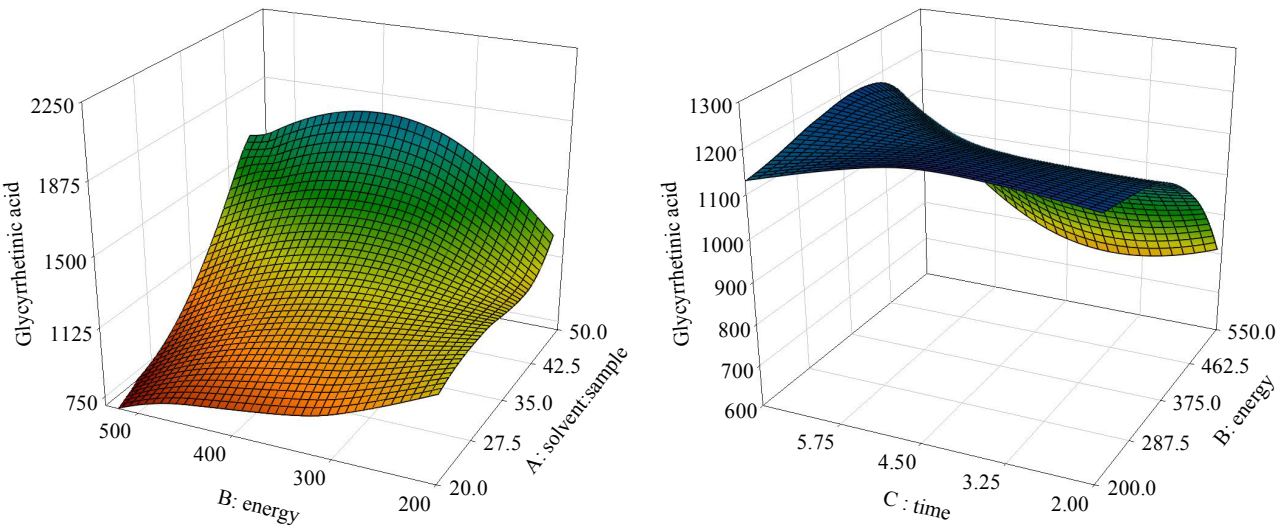


Figure 2. Response surface plots showing the effects of variables on value of GA: interaction of energy (B) and solvent:sample ratio (A); interaction of time (C) and energy (B obtained by microwave-assisted extraction. Results were obtained based on 3 replicates.

Due to the dielectric properties of the solvent and matrix, MAE heats the material by transferring heat from the surface to the centre of the material. Therefore, this technique is an alternative to other conventional methods used in plant material extraction (Das et al., 2013). Few prior studies have reported the extraction of GL, GA, CBX, and LQ from liquorice by using MAE. Pan et al. (2000) developed rapid and efficient MAE to retrieve GL from liquorice root but, compared with several conventional techniques, the recovery of GL was not significantly improved. Huang et al. (2014) determined the extraction efficiency of GL and LQ from dried liquorice plant by MAE using 50% methanol as the solvent. In that study, the best extraction efficiency was provided with a 1:100 solid-to-liquid ratio and 400 W of microwave power for 5 min, which resulted in average GL and LQ quantities of 44.26 ± 0.84 and 14.73 ± 0.17 mg g^{-1}, respectively. In comparison, the best yields in the current study were provided with a 1:10.09 solid-to-liquid ratio at 100.31 W for 7.00 min, and the corresponding concentrations of the 2 bioactive compounds were 120.4 and 15.3 mg g^{-1}, respectively. These analysis parameters and results were compared, and both solvent and energy savings, along with higher GL and LQ concentrations, are presented in the current study.

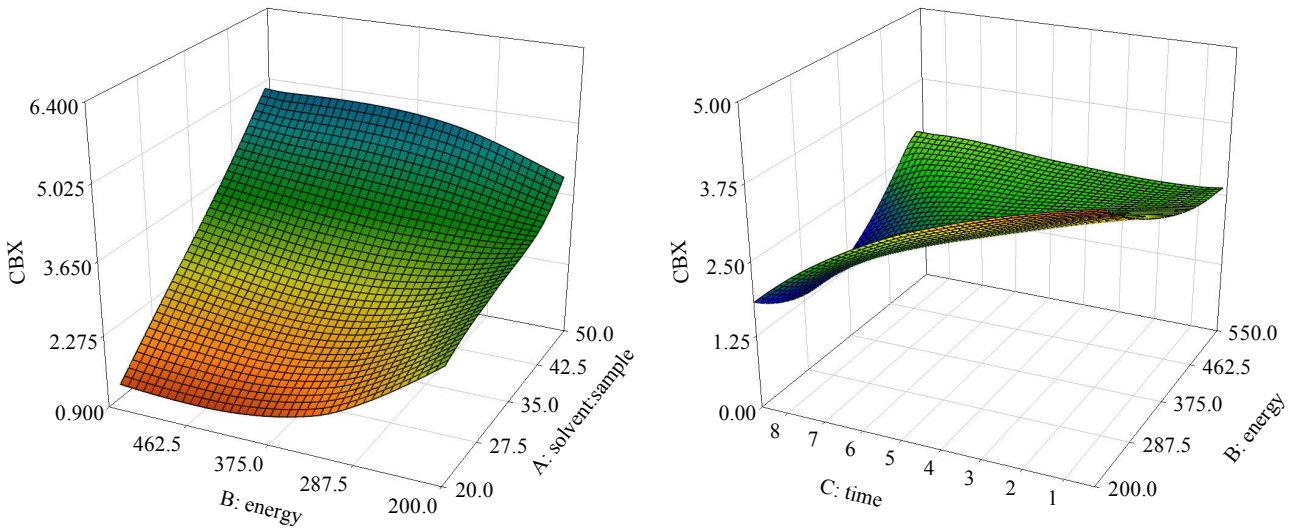


Figure 3. Response surface plots showing the effects of variables on value of CBX: interaction of energy (B) and solvent:sample ratio (A); interaction of time (C) and energy (B) obtained by microwave-assisted extraction. Results were obtained based on 3 replicates.

3.1.2. Ultrasound extraction using an ultrasonic processor

The ANOVA table of results for GL obtained by the RSM–CCD showed a model F-value of 69.96, meaning that the model was significant. The model terms A, B, C, BC, A^2, B^2, C^2, ABC, A^2B, A^2C, and AB^2 were significant. The lack-of-fit F-value of 0.36 was nonsignificant. Pred-R^2 was 0.8954 and adj-R^2 was 0.9792. The final equation for GL is described below:

$$Y1 = +42.44 + 2.07A + 6.49B + 2.51C - 0.77AB - 0.053AC + 4.51BC - 1.20A^2 - 1.50B^2 - 2.21C^2 + 1.82ABC - 8.19A^2B - 3.57A^2C + 4.81AB^2 \quad (5)$$

For GA, the ANOVA table indicated that the model was significant because the model F-value was 340.19. All of the model terms were significant. The lack-of-fit F-value of 1.02 was nonsignificant. Pred-R^2 was 0.9478 and adj-R^2 was 0.9957. For GA, the final equation was as follows:

$$Y2 = +3.51 + 0.57A - 1.06B + 0.31C + 0.72AB - 1.09AC + 1.15BC + 1.23A^2 + 0.12B^2 + 0.036C^2 - 0.55ABC + 0.30A^2B - 2.61A^2C + 0.75AB^2 \quad (6)$$

According to the ANOVA table for the CBX results, the model was significant. The significant model terms were A, B, BC, B^2, C^2, ABC, and AB^2. The lack-of-fit F-value of 0.06 was nonsignificant. Pred-R^2 was 0.9589 and adj-R^2 was 0.9671. For CBX, the final equation is provided below:

$$Y3 = +0.39 + 0.13A - 0.15B - 0.017C - 0.023AB + 0.036AC + 0.16BC - 1.955E - 003A^2 + 0.093C^2 + 0.064ABC + 0.033A^2B - 0.042A^2C + 0.11AB^2 \quad (7)$$

The model F-value of 15.16 for LQ demonstrated that the model was significant. The model terms AB, AC, BC, A^2, B^2, C^2, and AB^2 were significant. The lack-of-fit F-value of 0.02 was nonsignificant. Pred-R^2 was 0.9330 and adj-R^2 was 0.9065. For LQ, the final equation was as follows:

$$Y4 = +1.71 - 0.14A + 0.076B + 0.12C - 0.14AB - 0.21AC + 0.17BC - 0.11A^2 - 0.37B^2 - 0.35C^2 - 0.12ABC - 0.012A^2B - 0.040A^2C - 0.35AB^2 \quad (8)$$

The conditions for point prediction given by the RSM–CCD were 20 min of UAE at 55% amplitude and a 30.00 solvent-to-raw material ratio. Under these point prediction conditions, the maximal GL, GA, CBX, and LQ values attained were 42.44 mg g^{-1}, 3.51 μg g^{-1}, 0.39 μg g^{-1}, and 1.70 mg g^{-1}, respectively. According to the desirability function method, the optimum extraction conditions were 50.00 for the solvent-to-raw material ratio, 45.28% amplitude, and 7.43 min of UAE. The desirability value of the optimum extraction was 87.2%. At this value, the GL, GA, CBX, and LQ yields were 69.26 mg g^{-1}, 24.72 μg g^{-1}, 1.16 μg g^{-1}, and 1.48 mg g^{-1}, respectively. The yield of GL increased with increasing amplitude, while the yield of GA decreased. In addition, an increase of GA yield was observed with increase in extraction time. Furthermore, with an increase in solvent/sample ratio, an increase in yields of CBX and LQ was observed. This technique has great extraction efficiency due to the acoustic cavitation effects that occur in the solvent by ultrasonic waves, and this depends on ultrasonic properties, product characteristics, and ambient conditions. With increasing ultrasonic power, the cavitation effect increases the release of the analyte from the sample by providing better penetration of the solvent into the sample (Jang et al., 2017). On the other hand, ultrasonic power boosts can also hinder the release of bioactives from the cells, thus preventing an increase in yield during extended extraction (Vilkhu et al., 2008; Guo et al., 2019). Also, it may be related to the fact that bioactive compounds trigger degradation after excessively lengthened ultrasonic time

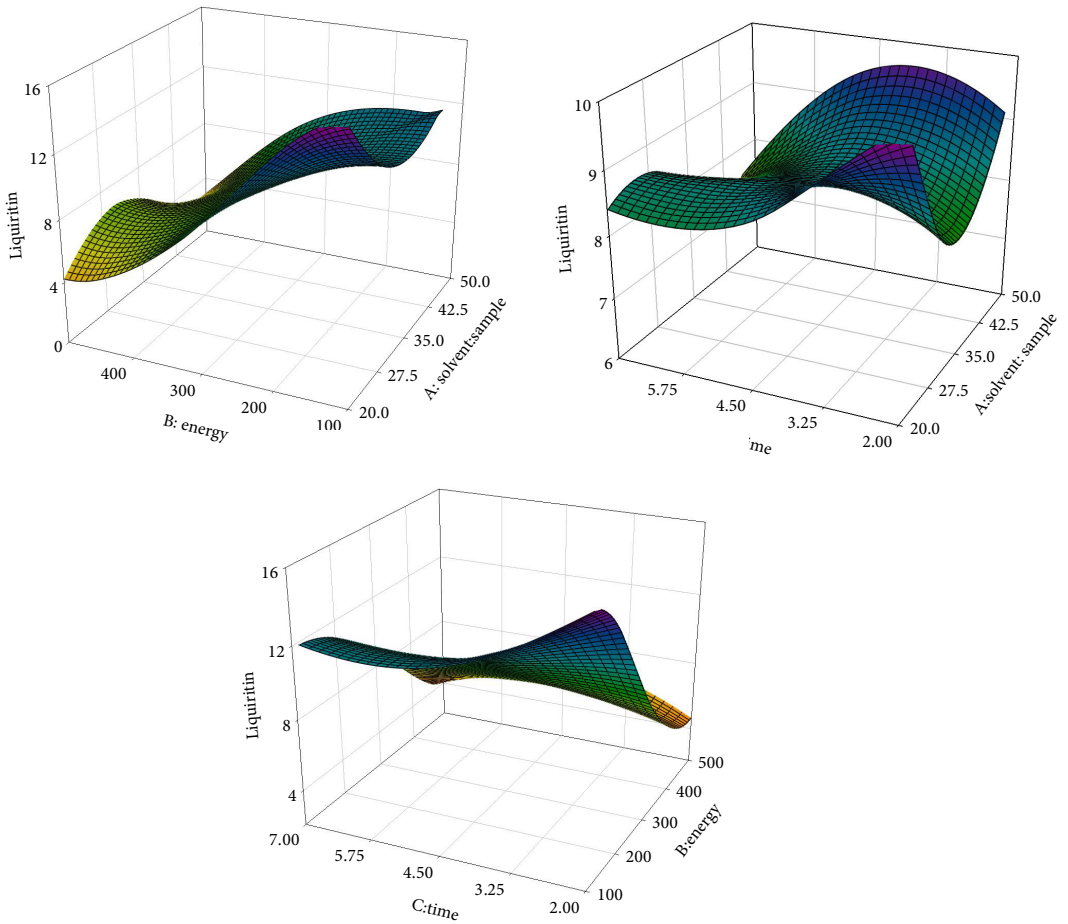


Figure 4. Response surface plots showing the effects of variables on value of LQ: interaction of energy (B) and solvent:sample ratio (A); interaction of time (C) and solvent:sample ratio (A); interaction of time (C) and energy (B) obtained by microwave-assisted extraction. Results were obtained based on 3 replicates.

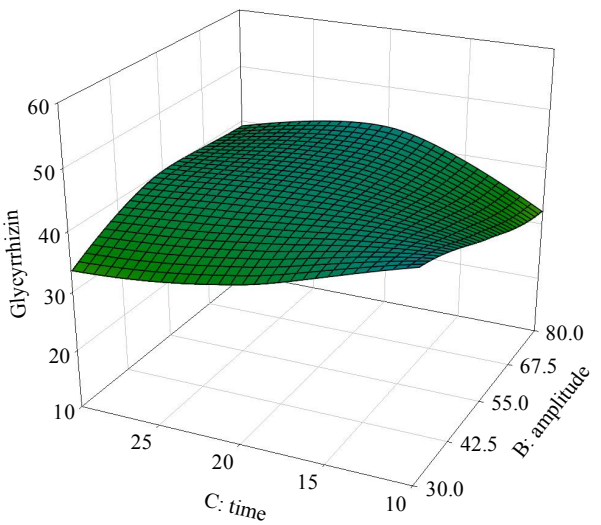


Figure 5. Response surface plots showing the effects of variables on value of GL: interaction of time (C) and amplitude (%) (B) obtained by ultrasound-assisted extraction. Results were obtained based on 3 replicates.

and therefore the yield of bioactives is decreased (Zhao et al., 2013). The literature reports that UAE requires less extraction time, lower temperature, and less solvent than conventional methods (Roselló-Soto et al., 2015; Chemat et al., 2017). Although it is a cheap and simple method, no current studies could be found about the extraction of GL, GA, CBX, and LQ from liquorice root using an ultrasonic processor. The present optimisation study is the first to be conducted using this technique.

3.1.3. Classical extraction

According to the ANOVA table of the GL results obtained by RSM–CCD, the model F-value of 592.40 showed that the model was significant. The model terms A, B, C, AB, AC, BC, A^2, C^2, ABC, A^2B, A^2C, and AB^2 were significant. The lack-of-fit F-value of 3.00 was nonsignificant. Pred-R^2 was 0.9350 and adj-R^2 was 0.9975. The final equation for GL is indicated as follows:

$$Y1 = +32.80 + 2.40A + 7.05B - 9.74C - 5.68AB - 3.31AC - 3.33BC - 0.64A^2 + 0.35B^2 + 3.71C^2 - 3.39ABC + 0.96A^2B + 4.79A^2C - 7.19AB^2 \quad (9)$$

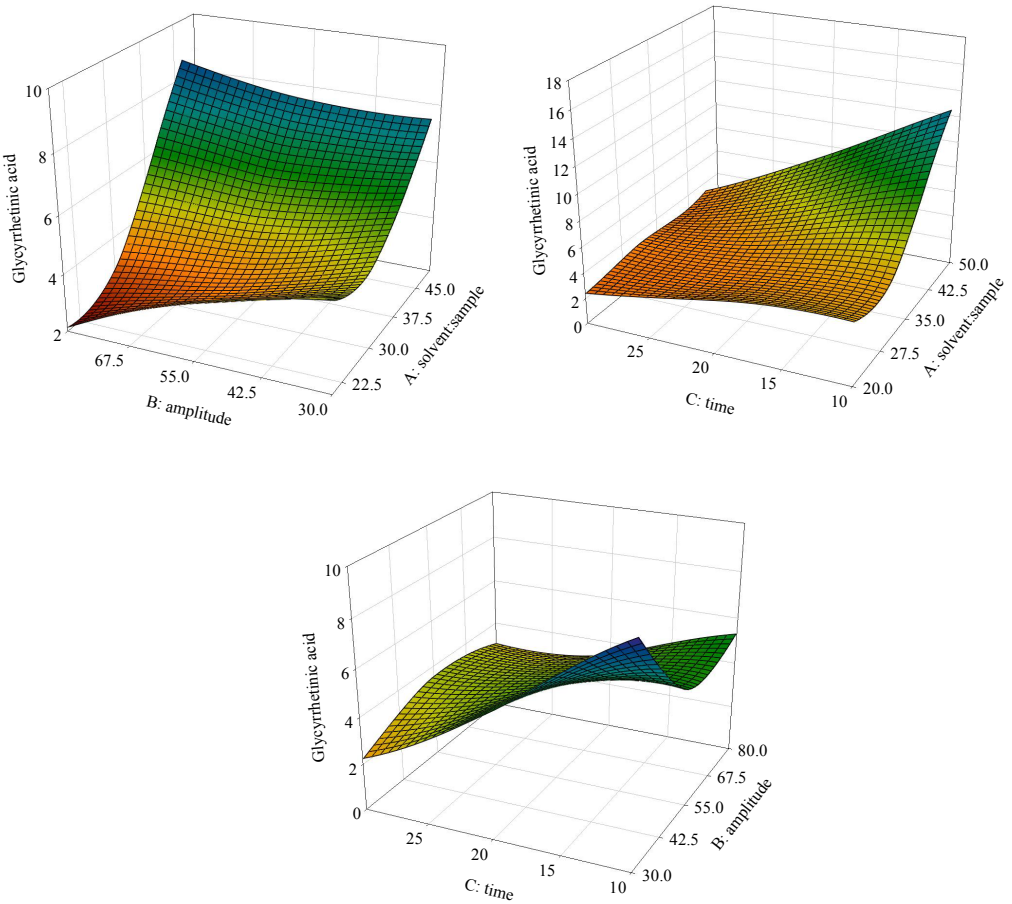


Figure 6. Response surface plots showing the effects of variables on value of GA: interaction of amplitude (%) (B) and solvent:sample ratio (A); interaction of time (C) and solvent:sample ratio (A); interaction of time (C) and amplitude (%) (B) obtained by ultrasound-assisted extraction. Results were obtained based on 3 replicates.

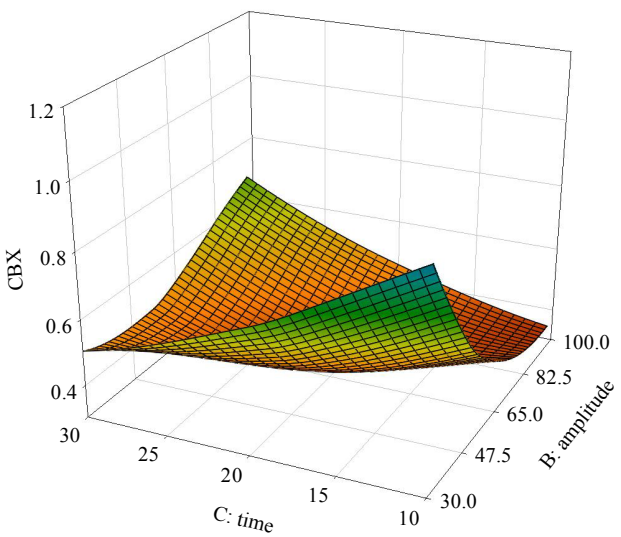


Figure 7. Response surface plots showing the effects of variables on value of CBX: interaction of time (C) and amplitude (%) (B) obtained by ultrasound-assisted extraction. Results were obtained based on 3 replicates.

For GA, the ANOVA table indicated that the model was significant because the model F-value was 6409.45. All of the model terms were significant. The lack-of-fit F-value of 0.98 was nonsignificant. Pred-R^2 was 0.9973 and adj-R^2 was 0.9998. The final equation for GA was as follows:

$$Y2 = +81.26 + 13.38A - 5.43B + 10.09C - 5.07AB + 5.53AC - 5.22BC - 17.75A^2 - 18.05B^2 - 10.20C^2 - 0.44ABC + 7.09A^2B - 6.82A^2C - 7.72AB^2 \quad (10)$$

The ANOVA table of the CBX results demonstrated that the model was significant (model F-value of 122.04). The model terms A, B, C, AB, AC, A^2, B^2, C^2, A^2B, and A^2C were significant. The lack-of-fit F-value of 0.66 was nonsignificant. Pred-R^2 was 0.8981 and adj-R^2 was 0.9881. For CBX, the final equation is provided below:

$$Y3 = +5.44 + 1.62A - 0.89B + 0.89C - 0.37AB + 0.18AC + 0.10BC - 0.17A^2 + 0.28B^2 + 0.33C^2 - 0.013ABC + 0.66A^2B - 1.23A^2C + 0.13AB^2 \quad (11)$$

The model F-value of 77.68 for LQ demonstrated that the model was significant. The significant model terms

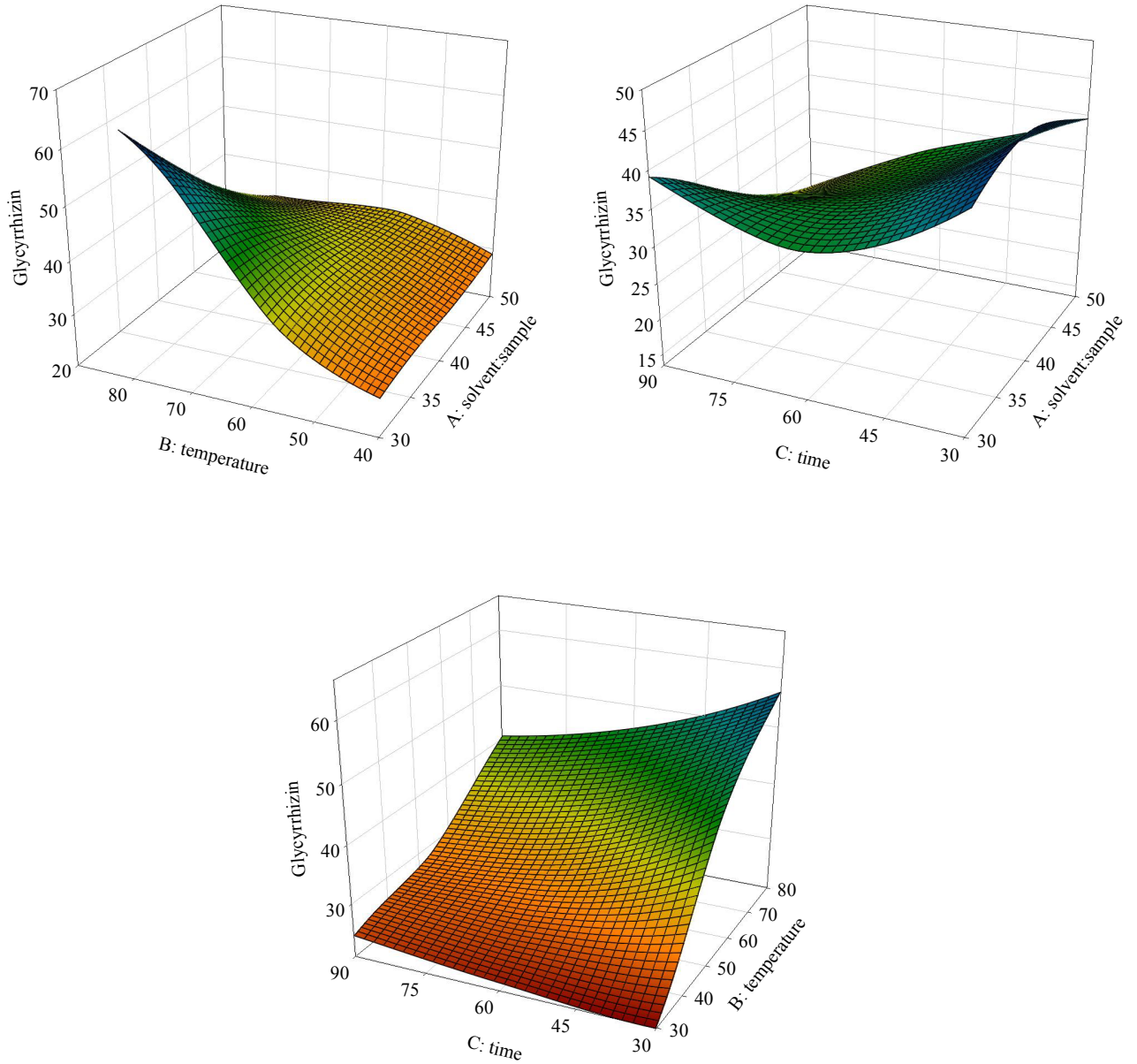


Figure 8. Response surface plots showing the effects of variables on value of GL: interaction of temperature (B) and solvent:sample ratio (A); interaction of time (C) and solvent:sample ratio (A); interaction of time (C) and temperature (B) obtained by classical extraction. Results were obtained based on 3 replicates.

were A, B, AB, AC, BC, A^2, B^2, C^2, ABC, A^2B, A^2C, and AB^2. The lack-of-fit F-value of 0.21 was nonsignificant. Pred-R^2 was 0.9388 and adj-R^2 was 0.9813. The final equation for LQ is given as follows:

$$Y4 = +5.13 - 0.53A + 0.65B + 0.24C + 1.18AB + 1.30AC + 1.08BC + 0.67A^2 - 0.56B^2 + 1.10C^2 + 1.27ABC + 0.73A^2B + 1.04A^2C + 0.73AB^2 \quad (12)$$

The conditions for point prediction given by the RSM–CCD were 60 min of extraction at 55 °C and a 40.00 solvent-to-raw material ratio. Under these point prediction conditions, the GL, GA, CBX, and LQ values from *G. glabra* reached 32.80 mg g^{-1}, 81.26 μg g^{-1}, 5.44 μg g^{-1}, and 5.12 mg g^{-1}, respectively. According to the desirability function method, the optimum extraction conditions were 20.00 for the solvent-to-raw material ratio and 80 °C and 10.00 min for classical extraction. The desirability value of the optimum extraction was 81.5%. At this value, the GL, GA, CBX, and LQ yields were 77.42 mg g^{-1}, 62.08 μg g^{-1}, 13.61 μg g^{-1}, and 9.44 mg g^{-1}, respectively. The yield of GL increased with increasing temperature, while the yield of CBX decreased. In addition, an increase of CBX yield and a decrease of GL yield were observed with an increase in

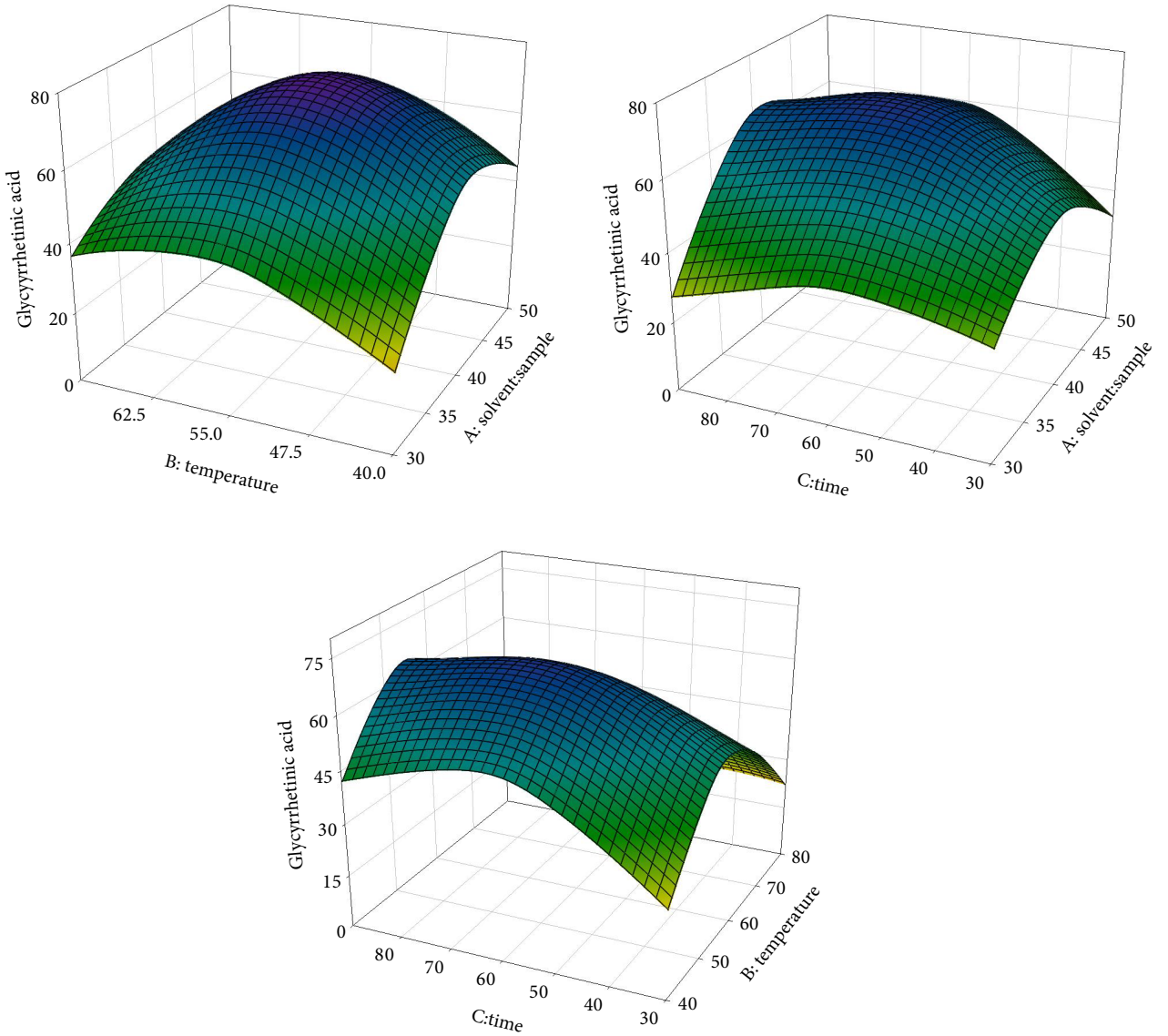


Figure 9. Response surface plots showing the effects of variables on value of GA: interaction of temperature (B) and solvent:sample ratio (A); interaction of time (C) and solvent:sample ratio (A); interaction of time (C) and temperature (B) obtained by classical extraction. Results were obtained based on 3 replicates.

extraction time. Furthermore, with an increase in solvent/sample ratio, an increase in the yields of CBX and GL was observed. High temperature can lead to the degradation of the interaction of solvent and sample while enhancing solvent diffusion rates (Richter et al., 1996). Therefore, the temperature of the extraction solvent should be kept in an appropriate range. Due to the fact that a long extraction time may lead to undesirable changes in the extracted compounds, the time should be optimised in a suitable range. On the other hand, it may enhance the yield of bioactives (Wen et al., 2018).

Wang and Yang (2007) extracted total GL, LQ, and GA amounts from *G. radix* in decreasing order of 40.733 ± 2.009 to 1.212 ± 0.054, 30.729 ± 1.552 to 0.451 ± 0.016, and 0.017 ± 0.00 to 0.605 ± 0.02 mg g^{-1}, respectively, from 20 liquorice samples by the classical extraction method at 60 °C for 6 h. The corresponding maximum values for these compounds in the present study were 60.0, 14.1, and 0.081 mg g^{-1} and these were obtained with the classical extraction method. Hennell et al. (2008) carried out classical extraction of liquorice root (*G. uralensis* Fisch. ex DC.) at 50 °C and 1:100 sample-to-solvent ratio for 2 h, which provided a GL yield of 24.95 mg g^{-1}. It appears that the present study is a more effective extraction method for GL than the above-mentioned studies. Furthermore, our study required less time for

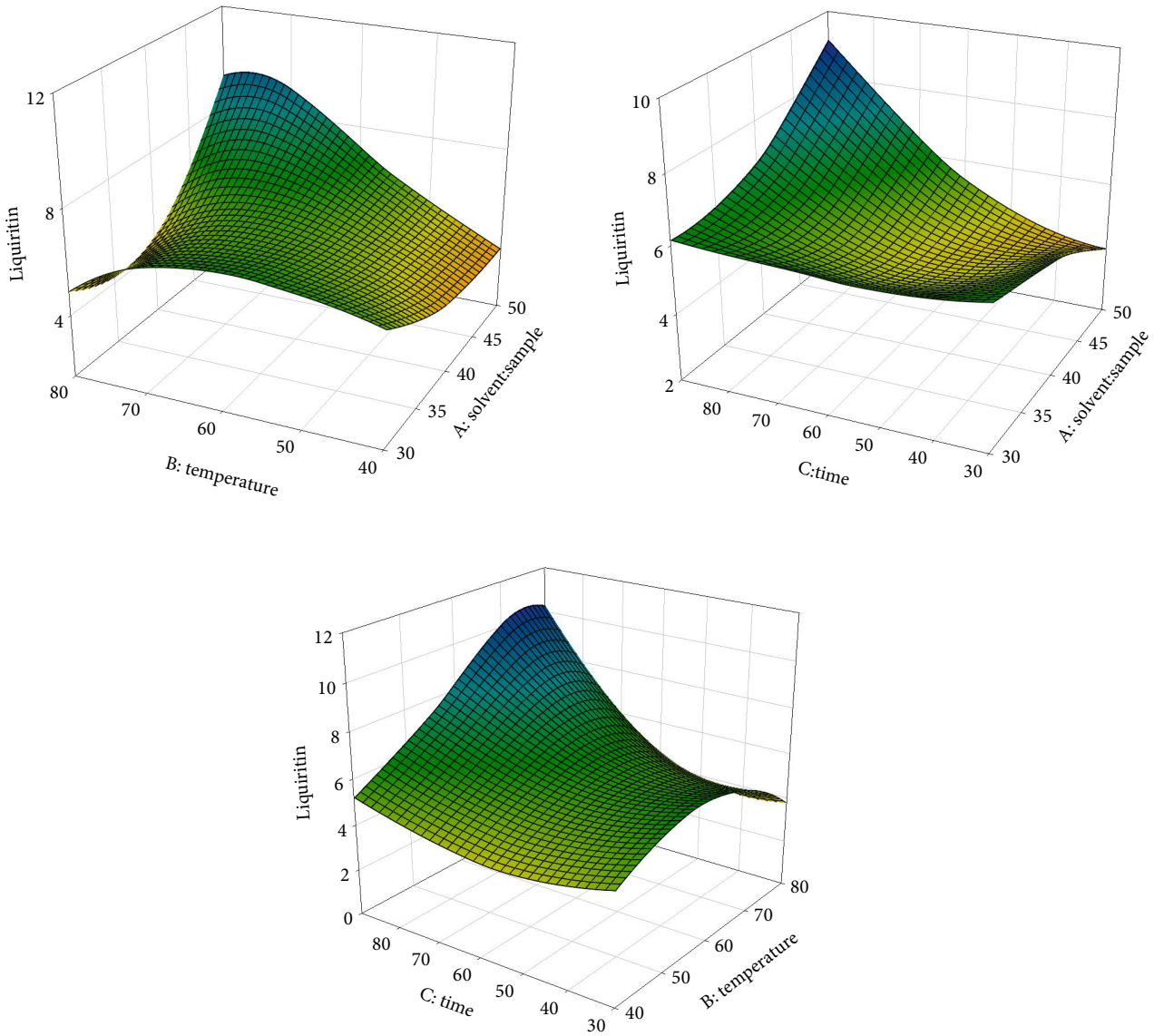


Figure 10. Response surface plots showing the effects of variables on value of LQ: interaction of temperature (B) and solvent:sample ratio (A); interaction of time (C) and solvent:sample ratio (A); interaction of time (C) and temperature (B) obtained by classical extraction. Results were obtained based on 3 replicates.

the extraction of these compounds compared to those found in the literature.

3.2. Verification tests

The verification tests were operated 3 times under point prediction conditions obtained by the RSM–CCD for all extraction methods. For MAE, the actual GL, GA, CBX, and LQ values were 76.2 ± 0.15 mg g^{-1}, 1156.2 ± 0.75 μg g^{-1}, 2.6 ± 0.12 μg g^{-1}, and 8.3 ± 0.06 mg g^{-1}, respectively (325 W of energy, 1:30 solid-to-liquid ratio for 4.50 min). For UAE, using the ultrasonic processor, the actual GL, GA, CBX, and LQ values were 61.4 ± 7.47 mg g^{-1}, 17.8 ± 3.45 μg g^{-1}, 0.99 ± 0.32 μg g^{-1}, and 1.4 ± 0.44 mg g^{-1}, respectively (45.30% amplitude, 1:50 solid-to-liquid ratio for 7.45 min). Finally, for the classical extraction method, the actual GL, GA, CBX, and LQ values were 69.7 ± 7.24 mg g^{-1}, 60.6 ± 8.08 μg g^{-1}, 12.0 ± 3.27 μg g^{-1}, and 10.2 ± 3.40 mg g^{-1}, respectively (1:20 solid-to-liquid ratio at 80 °C for 10 min).

3.3. Statistical analysis

The maximum mean values for GL (76.65 mg g^{-1}), GA (1138.84 μg g^{-1}), and LQ (8.52 mg g^{-1}) were obtained by MAE, whereas the maximum mean yield of CBX (5.75 μg g^{-1}) was achieved by the classical extraction method. The statistical results of the 4 bioactives extracted by the 3 extraction methods are shown in Table 5.

Table 5. Statistical results of 4 bioactives extracted by the 3 extraction methods ($P < 0.05$).

		N	Mean	Std. deviation	Std. error	95% confidence interval for Mean		Minimum	Maximum
						Lower bound	Upper bound		
Glycyrrhizin	Classic	20	35.1450	11.44182	2.55847	29.7901	40.4999	18.60	60.00
	UAE	20	39.0850	7.30402	1.63323	35.6666	42.5034	27.50	54.20
	Microwave	20	76.6500	14.94092	3.34089	69.6574	83.6426	52.00	120.40
Glycyrrhetinic acid	Classic	20	49.8600	25.03028	5.59694	38.1455	61.5745	8.70	81.80
	UAE	20	4.4600	2.49787	.55854	3.2910	5.6290	1.70	10.30
	Microwave	20	1138.8500	260.75282	58.30610	1016.8139	1260.8861	604.70	1783.40
Carbenoxolone	Classic	20	5.7500	1.69752	.37958	4.9555	6.5445	2.30	8.50
	UAE	20	.5500	.28191	.06304	.4181	.6819	.20	1.30
	Microwave	20	2.8350	1.13799	.25446	2.3024	3.3676	.90	4.90
Liquiritin	Classic	20	5.9650	2.41318	.53960	4.8356	7.0944	2.40	14.10
	UAE	20	1.1500	.52965	.11843	.9021	1.3979	.50	2.10
	Microwave	20	8.5100	2.86704	.64109	7.1682	9.8518	3.90	15.30

3.4. Conclusions

This study compared the extraction efficiency of 3 different techniques, including UAE, MAE, and classical extraction, to extract some bioactive and allergen compounds (GL GA, LQ, and CBX) from liquorice samples of *G. glabra* grown in Turkey. The parameters of the extraction methods were optimised by using the RSM–CCD approach. To establish the most efficient extraction technique, GL, GA, CBX, and LQ concentrations of each extract were determined by LC–MS/S and the results were analysed using one-way ANOVA. According to obtained results by LC–MS/MS, MAE was revealed as the most effective technique for isolating GL (mean: 76.65 mg g^{-1}), LQ (mean: 8.52 mg g^{-1}), and GA (mean: 1138.84 μg g^{-1}), while the classical technique was the most effective for CBX (mean: 5.75 μg g^{-1}). Regarding these bioactive compounds, the difference between the values of bioactive compounds obtained by these 3 extraction methods is thought to be due to the efficiency of the extraction methods. Extraction by ultrasound using the ultrasonic processor was the least effective technique for all 4 bioactive compounds. In contrast, MAE proved to be the fastest and most efficient method for extracting the bioactive compounds, except CBX. Furthermore, the GA yielded using microwave extraction was the highest reported for this approach in the literature to date. Extraction of all the above-mentioned bioactive compounds from liquorice root by comparing these 3 extraction methods in one study has not been observed in previous studies.

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