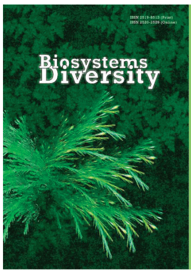


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# Optimum production conditions for the preparation of lemon balm (*Melissa officinalis*) tincture using response surface methodology

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Lemon balm (*Melissa officinalis*) is a member of medicinal and aromatic plants that have been used for phytotherapeutic applications for decades. In this study, optimum production conditions of lemon balm tincture, which is a functional product used commonly by the people for medicinal purposes, were investigated. For this aim, the response surface methodology approach was applied, and to determine the effect of processing variables, solid concentration ($X_1$), ethanol concentration ($X_2$), and processing time ($X_3$) were selected as the parameters showing the essential effects on the tincture production. The production of high phenolic yielded tincture was aimed at, and therefore, total phenolic content (TPC) and antiradical activity as inhibition concentration ($IC_{50}$) of the tinctures were determined as the responses in the study. According to the experimental design, 15 tincture samples were prepared, and TPC ranged 1,462–10,335 mg GAE/L, while the $IC_{50}$ ranged 11.8–78.1 mL. Multiple response optimization approaches were expertly applied, and optimum solid concentration and ethanol concentration for the high bioactive lemon balm tincture was determined as 5 g in 30 mL solvent and 38.6%, respectively. These optimum production conditions will help manufacturing the ideal lemon balm tincture with high bioactivity.

*Keywords*: tincture; bioactivity; optimization; aromatic plants; antiradical activity.

## Introduction

The tincture is a hydroalcoholic extract of various plants used for medicinal purposes, namely it is universally used in phytotherapy, and used effectively for the treatment of many diseases or reduction of fevers. Tinctures are the galenic pharmaceutical forms that are prepared by using hydroalcoholic extraction, and the tincture preparation methods using different medicinal plants are recommended by some Pharmacopoeias, e.g., European Pharmacopoeia and Polish Pharmacopoeia (Olech et al., 2012). In phytotherapeutic applications, the powder pharmaceutical forms of the plants are commonly used, but the liquid extracts of the plants called tincture have many advantages for the treatments of the diseases. Bone (2003) reported that the tinctures have superior bioavailability because all important phytochemical constituents are already present in the solution compared to solid dose preparation. Additionally, dose flexibility is easier for the herbal liquids, and children could ingest fluids easier compared to solids (Bone, 2003; Kaplan et al., 2019). Also, Sanchez Medina (2007) reported that the tincture products are popular because of availability, ease of dosage, and for convenient storage. Kowalczyk et al. (2012) reported that the tincture is a highly popular product because it is one of the most accessible forms of pharmaceutical formulation, and has a natural production process and use by the patients.

Lemon balm (*Melissa officinalis*), which belongs to the Lamiaceae family, is a perennial herb (Mirolieai et al., 2011; Lin et al., 2012). Radomir et al. (2019) reported that the lemon balm is a popular medicinal plant in traditional medicine due to its important pharmacological effects, used as extracts, tea, powder, tincture, infusion and decoction for internal use, and could be used externally such as in the forms of local baths, cataplasms, and gargle. Lemon balm is one of the popular medical plants exerting antioxidant (Lara et al., 2011; Spiridon et al., 2011), antimicrobial (Mimica-Dukic et al., 2004; Hancianu et al., 2008; Salamon et al., 2019; Zazharskyi et al., 2019), antiviral (Nolkemper et al., 2006; Sanchez-Medina et al., 2007) and anti-inflammatory (Bounihi et al., 2013) performance because of richness in many polyphenolic compounds. The response surface methodology (RSM) is a mathematical and statistical modeling approach used to facilitate calculations for the complex operations and to optimize the process parameters (Sheng et al., 2013; Tang & Choi, 2013; Wang et al., 2013). It is an important method to evaluate the multiple parameters and interactions effectively (Ma et al., 2010). In some studies, the response surface methodology approach was used successfully to determine the optimum processing conditions for the tinctures (Kaplan et al., 2018; Kaplan et al., 2019) and some important extract production (Zou et al., 2011; Köprü et al., 2020).

In the current research, the effects of the processing parameters, namely solid concentration, ethanol concentration and storage period on the bioactivity of lemon balm tincture were investigated and the production conditions were optimized using response surface methodology by using two important response parameters, in particular the total phenolic content and antiradical activity.

## Materials and methods

Lemon balm (*M. officinalis*) was provided as a dried form by the Boralife Medicinal Plant Company (Ankara, Turkey). The samples were ground using a miller and used as a powder form in the preparation of the tincture. For the preparation of the lemon balm tincture samples, the corresponding sample concentration (1–5 g) was weighed in a glass bottle, and 30 mL of ethanol in different concentrations (25–75%) was incorporated. Then, the mixture was mixed for a while by hand to ensure that the entire powder sample is soaked by the solvent. Finally, the bottles were stored at room temperature and in dark conditions for different processing periods (1–15 days). At the end of the storage period, the samples were filtered through filter paper. The filtrate called liquid tincture was used for the further bioactive analysis.

Total phenolic content (TPC) of the samples was determined using the method suggested by Singleton & Rossi (1965) after some minor modifications. For this purpose, 200 µL of the tincture was mixed with 1,800 µL of distilled water. Then, 1 mL of diluted (1/10) Folin Ciocelteau reagent was added, and 1 min later, 2 mL of sodium carbonate (2%

w/v) was poured into all tubes. Then, the samples were mixed for a while by vortex and incubated for 2 hours at room temperature and in dark conditions. At the end of the incubation, the absorbance values of the samples were recorded at 765 nm using a UV-vis spectrophotometer (Shimadzu, Japan). The total phenolic content of the samples was calculated as mg gallic acid equivalent GAE/L sample using the calibration curve. All measurements were performed in four replications.

DPPH radical scavenging activity test was followed as described by He et al. (2016). In this regard, to compare the radical scavenging performance of the samples, the values of inhibition concentration levels at a 50% ratio of DPPH radical for each tincture sample were calculated separately. For this purpose, each tincture sample was diluted at different levels, and all the diluted tinctures were exposed to DPPH radical scavenging test. Therefore, 100 μL of tincture sample was mixed with 3,900 μL of DPPH radical solution in methanol (2 mM) and mixed well-using vortex. After the incubation of the samples for 30 min in a dark place in room conditions, the absorbance values were recorded at 517 nm by a UV-vis spectrophotometer (Shimadzu, Japan). DPPH radical scavenging capacity was calculated as % inhibition using the following equation): % Inhibition = 100 – [($Abs_{control}$ –$Abs_{sample}$)/ $Abs_{control}$] x 100.

After the determination of the percentage inhibition of each tincture sample, the calibration curve was developed by using % inhibition and the tincture concentration levels. Then, the $IC_{50}$ value for each tincture samples was calculated using the calibration curve by plotting the percentage inhibition of DPPH as a function of the tincture concentration. All the measurements were performed for replications.

In the present study, response surface methodology based on Box-Behnken experimental design (Box & Behnken, 1960) was used for the determination of optimum tincture manufacturing process conditions for lemon balm plants. For this purpose, some basic and important processing factors, namely solid concentration ($X_1$), ethanol concentration ($X_2$), and storage period ($X_3$), were selected. Then, a 3-factor-3-level Box-Behnken experimental design having three replicates at the center point was developed using Design-Expert software (Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA). The experimental design showing the processing factors was created; their levels are presented in Table 1. As the responses, total phenolic content and antiradical activity were selected and the recorded mean values of the studied responses measured as the triplicate average were fitted to the second order polynomial model to understand the effect of the studied parameters on the selected response parameters as shown in Eq. 2.

$$Y - \varepsilon = \beta_0 + \sum_{i=1}^{N} \beta_i x_i + \sum_{i=1}^{N} \beta_{ii} x_i^2 + \sum_{\substack{i=1 \\ i<j}} \sum_{j=i+1} \beta_{ij} x_i x_j ,$$

where Y is the response value, $\beta_0$ is the intercept term, $\beta_i$ is the linear term, $\beta_{ii}$ is the quadratic term, $\beta_{ij}$ is the interaction term, and $X_i$ and $X_j$ are the coded levels of the independent variables. The regression coefficients were calculated also by using Design-Expert software ((Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA) for each of the analyzed responses and also all the computational work, including the designation of experimental points, randomization, and analysis of variance, fitting of the second-order polynomial models and graphical representations, as well as the optimization, was performed using the same software (Design-Expert® Software Version 7.0 (Stat-Ease Inc., Minneapolis, USA). Analysis of Variance (ANOVA) was applied to see the differences between the processing variables. Also, the determination of coefficients ($R^2$) was calculated, and the F values were computed to show the significance of the dependent variables ($P < 0.05$).

## Results

*Bioactivity of the tincture samples.* In the scope of the study, the best manufacturing conditions of the lemon balm tincture were determined in terms of some basic bioactive parameters. For this purpose, total phenolic content and radical scavenging activity of the samples were determined and subjected to the mathematical-statistical modeling using response surface methodology. Total phenolic content (TPC) of the tincture samples was in the range of 1,462–10,336 mg GAE/L. The highest TPC was determined for the sample which was prepared by 5 g of SC, 50% ethanol concentration stored for 15 days (run9) while the lowest one was determined in the tincture which was prepared by 1 g of SC, 25% ethanol concentration stored for eight days (run14). It was observed that the TPC of the tincture was affected by solid concentration significantly ($P < 0.05$, Table 3). The linear effect of EC was also found to be non-significant ($P < 0.05$), but the quadratic effect of the EC showed quite high significant effect on TPC of the tincture samples ($P < 0.01$, Table 3). Figure 1 illustrates the change in TPC of the tincture samples according to the processing variables. As indicated in Figure 1a and 1b, TPC of the tincture samples increased by the increase of solid concentration (SC). TPC of the samples changed slightly depending on the EC and it was observed that the excessive increase or decrease resulted in a decrement in TPC of the sample. Approximately, the highest TPC was recorded for the 50% of EC values (Fig. 1c and 1d). The effect of processing the time showed no significant effect on the TPC of the tincture samples ($P > 0.05$). As seen in Figure 1e and 1h, there was no clear and significant increase or decrease in the TPC of the samples in relation to the change in processing time ($P < 0.05$). The regression model developed to predict the TPC of the samples according to the processing variables showed that the model was fit a good prediction ($R^2 = 0.972$).

$$YTPC = –6438.8 + 2749.4X_1 + 303.5X_2 – 136X_3 – 14.7X_1X_2 + 6.8X_1X_3 + 2.7X_2X_3 - 78.4X_1^2 –3X_2^2 – 1X_3^2 \ (R^2 = 0.972)$$

**Table 1**
Box-Behnken experimental design showing the processing variables and their levels

| Runs | $X_1$ = SC, g | $X_2$ = EC, % | $X_3$ = time, day |
|---|---|---|---|
| 1 | 3 | 50 | 8 |
| 2 | 3 | 75 | 15 |
| 3 | 3 | 50 | 8 |
| 4 | 1 | 75 | 8 |
| 5 | 3 | 75 | 1 |
| 6 | 5 | 25 | 8 |
| 7 | 5 | 75 | 8 |
| 8 | 3 | 25 | 15 |
| 9 | 5 | 50 | 15 |
| 10 | 1 | 50 | 1 |
| 11 | 5 | 50 | 1 |
| 12 | 3 | 50 | 8 |
| 13 | 1 | 50 | 15 |
| 14 | 1 | 25 | 8 |
| 15 | 3 | 25 | 1 |

*Note*: SC – solid concentration, EC – ethanol concentration.

**Table 2**
Total phenolic content and antiradical activity of the tincture samples for each of the experimental runs

| Runs | SC, g | EC, % | Time, day | TPC, mg GAE/L | $IC_{50}$, mL |
|---|---|---|---|---|---|
| 1 | 3 | 50 | 8 | 6482 | 18.6 |
| 2 | 3 | 75 | 15 | 4272 | 31.5 |
| 3 | 3 | 50 | 8 | 6476 | 19.0 |
| 4 | 1 | 75 | 8 | 1969 | 65.3 |
| 5 | 3 | 75 | 1 | 3807 | 40.8 |
| 6 | 5 | 25 | 8 | 8273 | 16.4 |
| 7 | 5 | 75 | 8 | 5834 | 23.7 |
| 8 | 3 | 25 | 15 | 4552 | 30.8 |
| 9 | 5 | 50 | 15 | 10336 | 11.8 |
| 10 | 1 | 50 | 1 | 2314 | 55.3 |
| 11 | 5 | 50 | 1 | 9557 | 17.4 |
| 12 | 3 | 50 | 8 | 6814 | 18.1 |
| 13 | 1 | 50 | 15 | 2714 | 49.1 |
| 14 | 1 | 25 | 8 | 1462 | 78.1 |
| 15 | 3 | 25 | 1 | 5976 | 28.5 |

*Note*: SC – solid concentration, EC – ethanol concentration, TPC – total phenolic content, $IC_{50}$ – inhibition concentration for 50% of DPPH radical.

Radical scavenging activity of the samples was determined as percentage inhibition and also to compare the samples, $IC_{50}$ values were calculated for each tincture sample using the calibration curve developped by the percentage inhibition against the TPC of the diluted samples. Then, the tincture sample required to scavenge the 50% of DPPH was calculated using the TPC values, which were determined from the curve. $IC_{50}$ is a widely used parameter showing the concentration of antioxidants needed to decrease the initial DPPH concentration by 50% and it is known that a lower $IC_{50}$ value shows the higher antiradical performance for the related sample. As demonstrated in the table, the highest $IC_{50}$ value was calculated as 78.1 mL for the tincture which was prepared by 1 g of SC and 50% EC for the eight storage days while the lowest $IC_{50}$ value was seen for the sample prepared using 5 g of SC and 50% EC at 15 days storage (11.8 mL). Similar to the TPC of the samples, the linear effect of the SC also showed a significant effect on $IC_{50}$ values of the samples (P < 0.05, Table 4).

**Table 3**
ANOVA table for the response of total phenolic content

| Source | Sum of Squares | df | Mean Square | F Value | p-value Probability > F |
|---|---|---|---|---|---|
| Model | $1.0 \cdot 10^8$ | 9 | 11157292 | 19.4 | 0.002 |
| $X_1$-SC, g | $8.2 \cdot 10^7$ | 1 | 81539901 | 141.9 | < 0.0001 |
| $X_2$-EC, % | $2.4 \cdot 10^6$ | 1 | 2399974 | 4.2 | 0.096 |
| $X_3$-Time, day | $6.1 \cdot 10^3$ | 1 | 6102 | 0.0 | 0.922 |
| $X_1X_2$ | $2.2 \cdot 10^6$ | 1 | 2169649 | 3.8 | 0.110 |
| $X_1X_3$ | $3.6 \cdot 10^4$ | 1 | 35793 | 0.1 | 0.813 |
| $X_2X_3$ | $8.9 \cdot 10^5$ | 1 | 891621 | 1.6 | 0.268 |
| $X_1^2$ | $3.6 \cdot 10^5$ | 1 | 363506 | 0.6 | 0.463 |
| $X_2^2$ | $1.3 \cdot 10^7$ | 1 | 13221611 | 23.0 | 0.005 |
| $X_3^2$ | $8.1 \cdot 10^3$ | 1 | 8055 | 0.0 | 0.910 |
| Residual | $2.9 \cdot 10^6$ | 5 | 574663 | – | – |
| Lack of Fit | $2.8 \cdot 10^6$ | 3 | 932905 | 25.0 | 0.039 |
| Pure Error | $7.5 \cdot 10^4$ | 2 | 37299 | – | – |
| Corrected Total | $1.0 \cdot 10^4$ | 14 | – | – | – |
| $R^2$ | 0.972 | – | – | – | – |
| Adjusted $R^2$ | 0.922 | – | – | – | – |

*Note*: SC – solid concentration, EC – ethanol concentration.

The linear effect of EC and processing time did not show a significant effect, but their quadratic effect was found to be significant (P < 0.05, Table 4). The changes in $IC_{50}$ values of the samples are illustrated in Figure 2. As can be seen clearly, the increase in SC values caused a clear decrease in $IC_{50}$ values of the samples (Fig. 2a, 2b). It means that the increase in the solid level of the tincture formulation provided a lower tincture amount to scavenge the 50% of the DPPH radical. Figure 2c and 2d also showed the effect of EC on the $IC_{50}$ values, and as can seen, after the constant ethanol concentration value, the response of $IC_{50}$ showed an increase or decrease. Regarding the effect of the processing time, it did not show a clear effect on the $IC_{50}$ values of the samples (Fig. 2e and 2f). According to the recorded results for both responses, negative and significant correlation between TPC and $IC_{50}$ was determined (r = –0.906, P < 0.05).

**Table 4**
ANOVA table for the response of $IC_{50}$

| Source | Sum of Squares | df | Mean Square | F Value | p-value Probability > F |
|---|---|---|---|---|---|
| Model | 5452.5 | 9 | 605.8 | 20.95 | 0.002 |
| $X_1$-SC, g | 3981.2 | 1 | 3981.2 | 137.7 | < 0.0001 |
| $X_2$-EC, % | 7.0 | 1 | 7.0 | 0.241 | 0.644 |
| $X_3$-Time, day | 43.9 | 1 | 43.9 | 1.517 | 0.273 |
| $X_1X_2$ | 101.6 | 1 | 101.6 | 3.513 | 0.120 |
| $X_1X_3$ | 0.1 | 1 | 0.1 | 0.003 | 0.955 |
| $X_2X_3$ | 34.5 | 1 | 34.5 | 1.193 | 0.325 |
| $X_1^2$ | 714.2 | 1 | 714.2 | 24.7 | 0.004 |
| $X_2^2$ | 664.8 | 1 | 664.8 | 22.99 | 0.005 |
| $X_3^2$ | 3.3 | 1 | 3.3 | 0.116 | 0.748 |
| Residual | 144.6 | 5 | 28.9 | – | – |
| Lack of Fit | 144.1 | 3 | 48.0 | 209.5 | 0.005 |
| Pure Error | 0.5 | 2 | 0.2 | – | – |
| Corrected Total | 5597.0 | 14 | – | – | – |
| $R^2$ | 0.974 | – | – | – | – |
| Adjusted $R^2$ | 0.928 | – | – | – | – |

*Note*: SC – solid concentration, EC – ethanol concentration.

The constructed regression model for $IC_{50}$ values according to the processing variables showed that the model was fit a good prediction ($R^2$ = 0.97).

$$Y_{IC50} = –147.7 – 37.2X_1 – 2.28X_2 + 0.1X_3 + 0.1X_1X_2 + 0.01X_1X_3 – – 0.02X_2X_3 + 3.5X_1^2 + 0.02X_2^2 + 0.02X_3^2 \ (R^2 = 0.974)$$

*Multiple response optimization using desirability function.* To determine the best processing conditions using three basic process parameters namely solid concentration (SC), ethanol concentration (EC) and processing time, multiple response optimization procedures were applied. In this method, the most optimum process parameter levels were calculated. For this purpose, maximization and minimization applications were conducted, and so, the minimum and maximum response values and their correspondence parameters were calculated. Figure 3 illustrates the calculated response values for each response parameter.

Due to the negative correlation between TPC and $IC_{50}$, in the application of maximization, the criteria were selected as maximum for TPC and minimum for $IC_{50}$ and vice versa. Maximization results showed that the maximum TPC (9707.7 mg GAE/L) and also minimum $IC_{50}$ values (11.83 mL) would be at SC = 5 g, EC = 38.6%, and processing time = 1.44 days. It was observed that the solid concentration is quite important for the highly yielded bioactive compounds because the increase in SC provided high TPC in the final product. Therefore, when the SC was used as the maximum, processing time was shorter. Minimization results revealed that the minimum TPC (418.6 mg GAE/L) and maximum $IC_{50}$ (73.66 mL) would be at SC = 1 g, EC = 25%, and processing time = 15 days. As seen, the decrease of SC caused a decrement in TPC and also a big increase for the tincture amount to provide the scavenging of the DPPH at 50%. The desirability function for both maximization and minimization was calculated as 0.964 and 0.966, respectively. It was concluded from the multiple response optimization procedures that the maximum bioactivity for the lemon balm tincture could be obtained by using maximum solid and moderate ethanol concentration.

## Discussion

Tincture production conditions of lemon balm were investigated, the manufacturing process parameters were optimized using two important responses such as total phenolic content and antiradical activity using response surface methodology. Regarding the TPC of the lemon balm tincture in the present study, similar and also parallel results about the effect of processing variables on TPC of the samples was reported by Radomir et al. (2019) who investigated the effect of some processing variables of microwave extraction on total polyphenol content of the lemon balm tincture. In this study, the effect of ethanol in the concentrations of 96% and 70% were investigated, determining that the 70% ethanol showed better polyphenol extraction in the tincture production using lemon balm plant. In some researches, the effect of processing time (extraction time) was also studied, and it was reported that the TPC of the tincture was recorded as 3.41 mg tannic acid equivalent (TAE)/mL after 5 min. When the extraction time increased by 15 min, TPC of the samples was measured as 1.72 mg TAE/mL. Radomir et al. (2019) revealed that the prolonged extraction time for lemon balm tincture did not increase the TPC of the final product; on the contrary, a significant decrease for TPC was observed. Vongsangnak et al. (2004) reported that an increased extraction period could take negative effects such as degradation or transformation of the phenolic compounds. Chan et al. (2009) stated that the longer extraction time is not recommended because prolonging the extraction process may lead to oxidation of phenols due to exposure to light or oxygen. Fick's second law implies that the final equilibrium between the solid level and liquid level is achieved after a certain time, which means that the excessive processing period will not show any recovery of the extracted compounds (Chan et al., 2009).

In the present study, maximum TPC values were recorded for the hydroalcoholic solvent, which means that the mixture of ethanol and water in the determined concentration showed the highest bioactivity. Similar results were reported by Kaplan et al. (2019) who stated that the maximum bioactive performance for the origanum tincture was achieved by moderate ethanol concentration (50%) at the stable: liquid ratio of 1:5. For the ethanol concentration to be used in the high bioactive lemon balm tincture, it was suggested that the EC should range 45% to 25% (Sanchez-Medina et al., 2007) as similar to the calculated EC value in the present work. Thoo et al. (2010) reported that binary solvent system was superior compared to mono-solvent system for the increase of extraction yield of phenolic compounds and the preservation of their relative polarity.

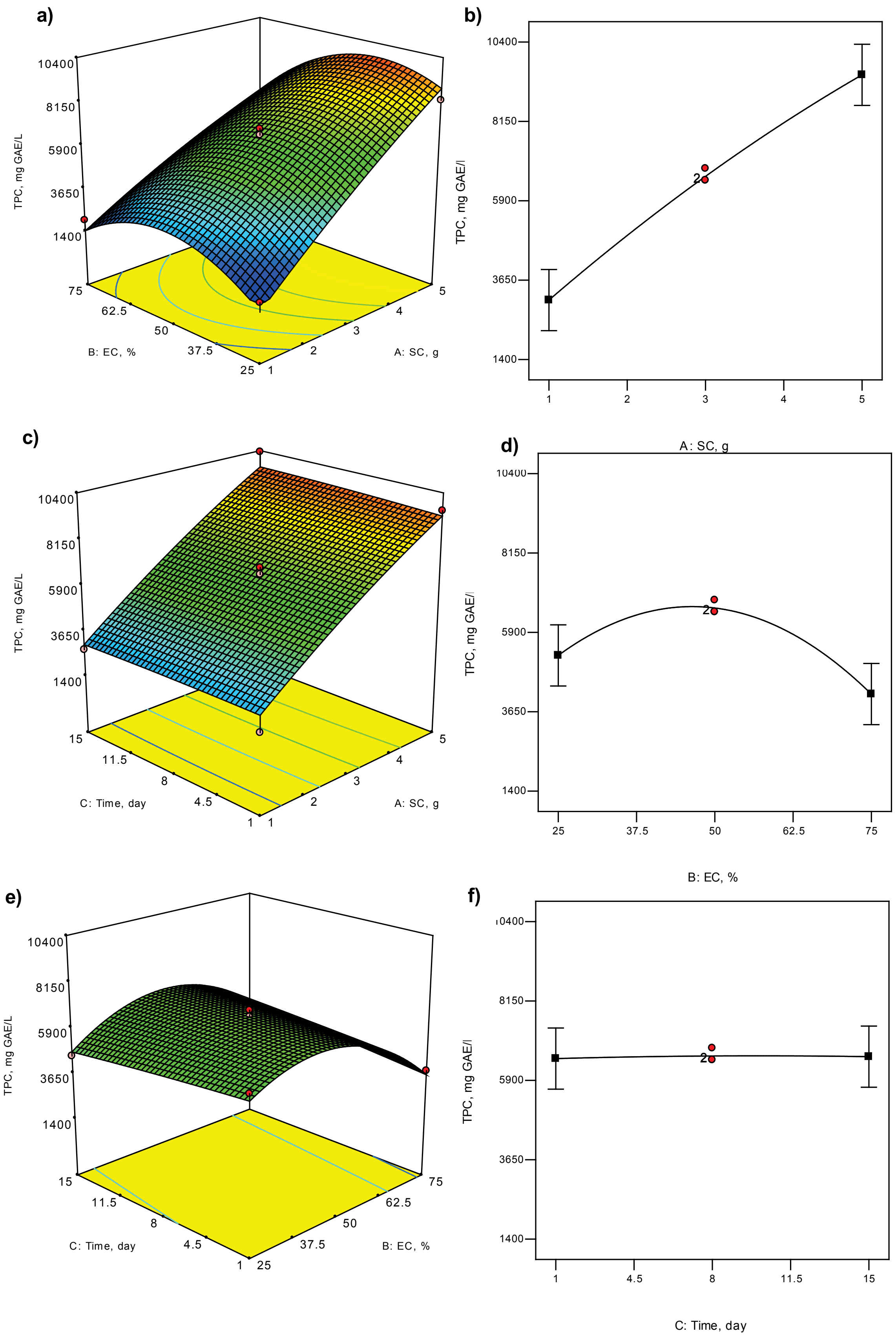


**Fig. 1.** Change in total phenolic content (TPC) of the tincture samples according to the processing variables: SC – solid concentration, EC – ethanol concentration

The results of the present work revealed that there was a significant and negative relationship between the TPC and $IC_{50}$ of the tincture samples, meaning that the increased TPC decreased the tincture amount to be consumed for the scavenging of 50% of the DPPH radicals. Similarly, Brighente et al. (2007) performed a research about the bioactive properties of some herbs and reported a negative relationship between the total phenolic content of the analyzed herbs and their $IC_{50}$ values.

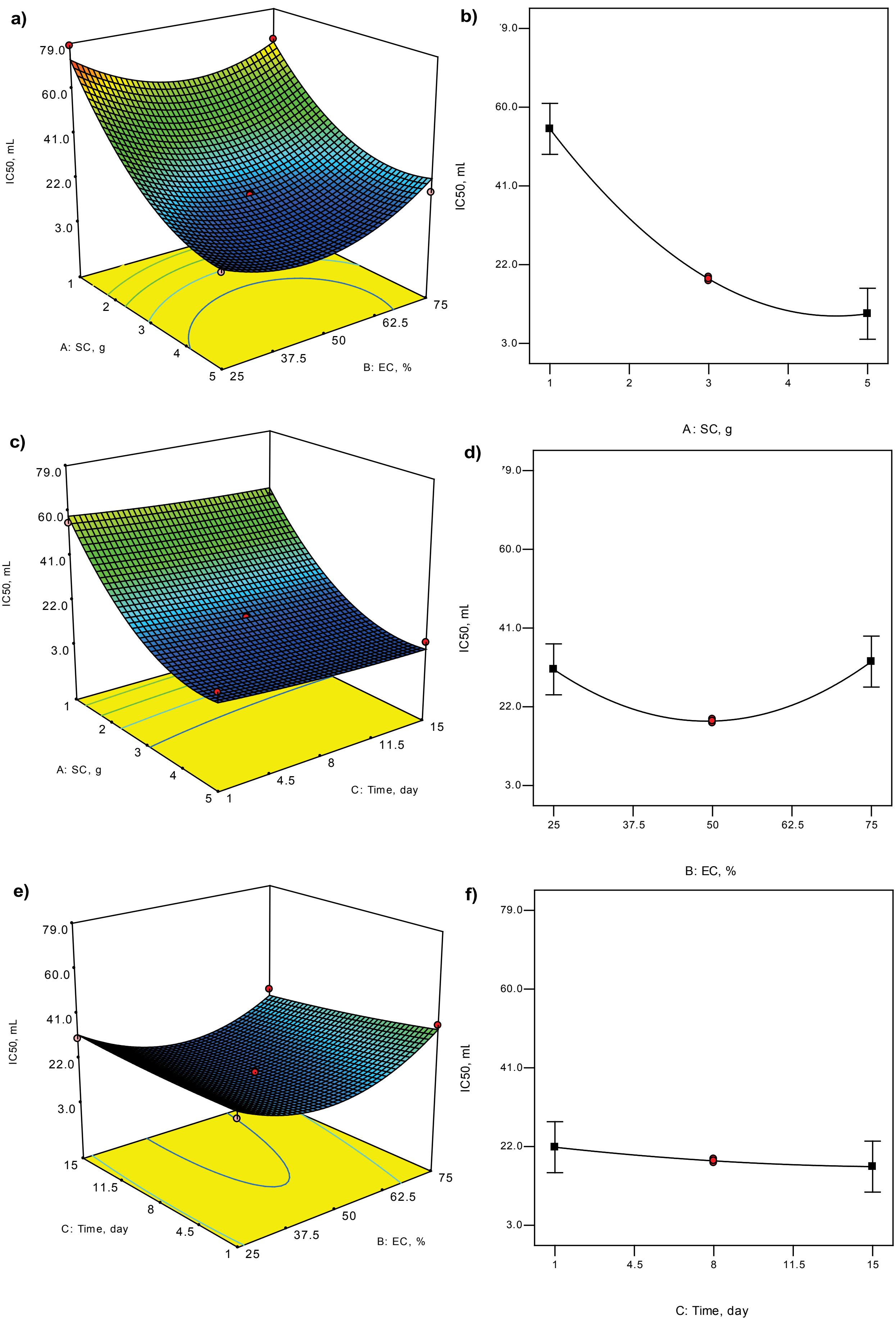


**Fig. 2.** Change in inhibition concentration for 50% of DPPH radical ($IC_{50}$) of the tincture samples according to the processing variables: SC – solid concentration, EC – ethanol concentration

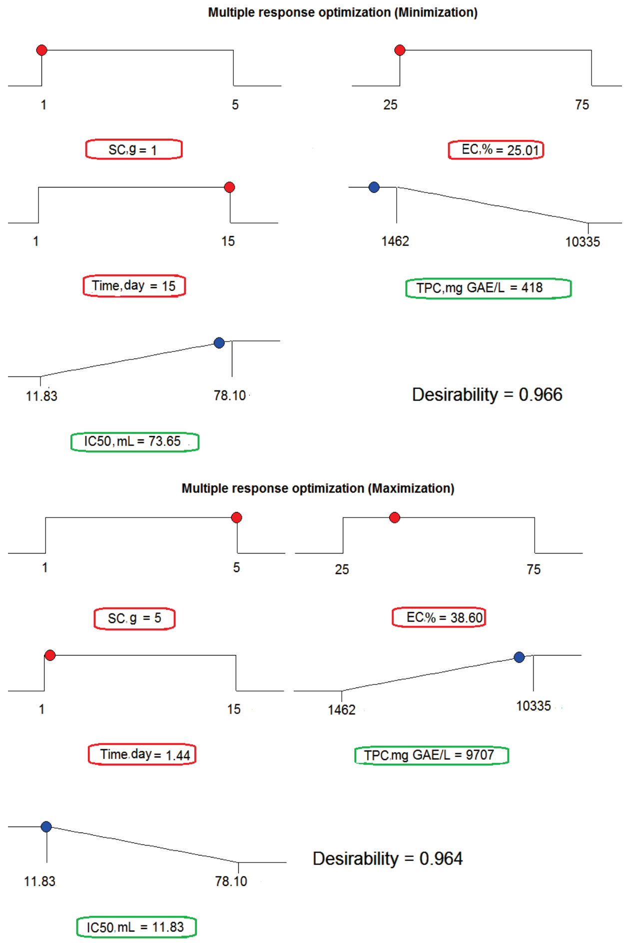


**Fig. 3.** Multiple response optimization to determine the best processing conditions for the tincture production: SC – solid concentration, EC – ethanol concentration, TPC – total phenolic content, $IC_{50}$ – inhibition concentration for 50% of DPPH radical

## Conclusion

To produce lemon balm tincture having high bioactivity, response surface methodology, which is a simplified mathematical-statistical modeling approach, was efficiently used to determine the best production conditions. It was determined that the solid material corresponding to the amount of lemon balm plant showed a significant effect on the bioactivity of the final tincture sample in terms of total phenolic content and antiradical activity. The best solvent was determined as the hydroalcoholic solvent because the full phenolic recovery from the plant tissues decreased by using sole ethanol or water as an extraction solvent. Multiple response optimization approaches were also used successfully, and therefore, by using both total phenolic content and radical scavenging capacity of the samples, the optimum tincture conditions were determined. The recorded optimum production conditions would be useful for the tincture industry to prepare more effective and higher bioactive tinctures for the consumers.

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