Extracting the antioxidants from *Nigella sativa* seeds using response surface methodology

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Abstract

Although many studies have explored the bioactive compound content of *Nigella sativa* (black seed – BS) essential oil, data on the phenolic compound content in the extracts of *Nigella sativa* seeds gives inconclusive results [1–3]. In this study, response surface methodology (RSM) with a central composite plan (CCP) was used for the experimental design and identification of the optimal conditions for maximizing the phenolic content and the antioxidant activity of BS extract. The independent variables were as followed: temperature (22.9–67.1°C), time (31.7–208.2 min), and methanol concentration in water (5.9–94.1% v/v). Different response variables (total polyphenols – TPC, total flavonoids – TFC and the antioxidant activity – DPPH and TEAC) required different optimal conditions to maximize them. The multi-response optimization was performed to determine the extraction conditions, which would simultaneously ensure the highest phenolic content and the antioxidant activity. The optimal conditions derived from the multi-response desirability function were as followed: 120 min, 56 °C and 50% methanol, giving the TPC = 7.05 mg/g, TFC = 3.05 mg/g, DPPH = 9.04 µmol/g and TEAC = 33.24 µmol/g (with desirability function coefficient = 0.83). Solvent concentration was established as a crucial parameter for the extraction of antioxidants from BS. A quercetin derivative and two derivatives of kaempferol were determined by HPLC (high performance liquid chromatography) analysis of the BS extract at the optimal conditions. The accuracy of the models for all response variables was confirmed by a high correlation coefficient ($r = 0.99$) between the experimental values and those predicted under optimal conditions.

Keywords Black seed · *Nigella sativa* · Extraction optimization · Kaempferol · Quercetin · Response surface method

Introduction

*Nigella sativa*, also known as black seed (BS) or black cumin, is an aromatic spice cultivated in Mediterranean regions, Asia and Africa. The plant is also cultivated in southern Poland [4]. The seeds are slightly bitter and peppery in taste and could substitute black pepper as a food seasoning. The spice is often added to cheese, bread and meat. BS is mainly known a source of edible oil, which constitutes around 30-50% of the seed mass [4]. BS oil includes mainly unsaturated fatty acids, sterols, tocopherols and other vitamins, and thus it is used in cosmetics and dietary supplements. BS has been a part of ancient and traditional medicine as a remedy for various illnesses such as chronic cough, asthma and eczema [5]. It possess a wide spectrum of pharmacological properties including gastroprotective, hepatoprotective, nephroprotective, neuroprotective, anti-inflammatory, antimicrobial, antiviral, and antioxidant [5–8]. Most of these positive health effects are ascribed to thymoquinone (TQ), a C10 terpene predominantly found in the oil fraction of BS [9]. TQ has been shown to exert high antioxidant activity as hydroxyl radical and hydrogen peroxide scavengers [10]. However, apart from TQ, other bioactive compounds can also be found in BS, including coumarins, flavonoids like quercetin, kaempferol or various phenolic acid [1–3, 5, 11–16], all of which are potent antioxidant active agents. The phenolic profile of BS extract...
results from the extraction procedure. Although various extraction techniques have recently been introduced, such as microwave-assisted extraction, ultrasound-assisted extraction, enzymatic-assisted extraction, pulsed electric field extraction, supercritical fluid extraction, natural deep eutectic solvent extraction, and accelerated solvent extraction [15, 17, 18], solvent extraction remains the most popular method [1, 3, 11, 12]. Recently, it has been reported that various Ethanolic extracts from black seed incorporated into minced chicken, minced pork or minced beef meat delayed the oxidative changes of lipids and or proteins, although BS extracts showed low antioxidant activity when comparing to other spices [11, 13, 19–21]. Thus, it is interesting to determine the optimal conditions for the extraction of bioactive compounds from BS in order to maximize its antioxidant activity and phenolic content. Many studies have explored the bioactive compound content of BS essential oil and its pro-health properties, whereas data on the phenolic compound content in BS seeds gives inconclusive results, which depend greatly on the extraction conditions (time, temperature and solvent type).

Thus, the aim of the study was to optimize the conditions (time, temperature and methanol concentration) for the maximal antioxidant activity and or phenolic compounds in extracts from BS using single- and multi-response surface methodology (RSM) with a central composite plan (CCP) for experimental design and model fitting. Additionally, HPLC analysis of BS extracts prepared in the optimal conditions was performed in order to identify the individual phenolic compounds.

Materials and methods

Chemicals

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu phenol reagent (FCR), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid and quercetin were supplied from Sigma-Aldrich (Steinheim, Germany). Aluminum chloride (AlCl3), sodium chloride (NaCl), potassium dihydrogen phosphate (KH2PO4), disodium hydrogen phosphate dodecahydrate (Na2HPO4·12 H2O), potassium chloride (KCl), sodium carbonate (NaOH), methanol, were obtained from POCh (Gliwice, Poland). The kaempferol to be used as the standard was from Fluka (Buchs, Switzerland). Organic solvents of HPLC grade were also purchased from Sigma-Aldrich (Steinheim, Germany).

Extract preparation

*Nigella sativa* dried seeds were purchased from a local distributor. They were collected in the same year as the study was performed. The seeds were ground in an electric mill, and then a 2 g portion of seeds was covered with 30 ml of extraction solvent and shaken in a water bath in controlled temperature and time conditions. Extraction conditions: time, temperature and concentration of methanol were determined based on a preliminary study using the experimental design generated by Statistica 13.3 software StatSoft Inc. (Tulsa, OK, USA). Then, the extracts were separated from solids and filtered through 13 mm, 0.45 μm PTFE syringe filters (Waters) into vials, and kept in a deep freeze at -80°C until analysis was conducted.

Experimental design

RSM with CCP was used for the experimental design and identification of the optimal conditions to maximize the phenolic content and radical scavenging activity of black seed. The complete factorial design consisted of eight cube (factorial) points, six star points and five center points. The whole experiment was performed in 3 replicates, thus the total number of runs was 57, including 15 center points. The effects of unexplained variability in the observed response due to extraneous factors were minimized by randomizing the order of the experimental runs. The independent variables were as followed: temperature (*T*, 22.9–67.1°C), time (*t*, 31.7–208.2 min), and methanol concentration in water (*c*, 5.9–94.1% v/v), while response variables were as followed: total polyphenol content (TPC), total flavonoid content (TFC) and the radical scavenging activity measured by the DPPH and the TEAC methods. Independent variables were coded at five levels: −1.47, −1, 0, 1, 1.47 (Table 1).

The second-order polynomial model was fitted to the data, by the equation:

\[
Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j
\]

where *Y* is the response (TPC, TFC, DPPH or TEAC), \(\beta_0, \beta_i, \beta_{ii}, \beta_{ij}\) are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and \(X_i, X_j\) are the independent variables (*T*, *t* or *c*).

TPC (total polyphenol content)

TPC was determined spectrophotometrically according to the method presented by Singleton and Rossi [22]. An
Table 1: The RSM CCP (response surface methodology with a central composite plan) design with uncoded and coded (in brackets) process variables and experimentally obtained responses for black seed (*Nigella sativa*) extracts.

| Runs | Temperature ($^\circ$C) | time (min) | Methanol concentration (%) | TPC (mg GAE/g) | TFC (mg QE/g) | TEAC (µmol/g) | DPPH (µmol TE/g) |
|------|------------------------|------------|-----------------------------|----------------|---------------|---------------|-----------------|
| 1    | 22.9 (-1.47)           | 120 (0)    | 50 (0)                      | 4.23 ± 0.38    | 2.47 ± 0.08   | 30.53 ± 0.19  | 6.74 ± 0.08     |
| 2    | 30 (-1)                | 60 (-1)    | 20 (-1)                     | 6.34 ± 0.35    | 2.03 ± 0.06   | 29.85 ± 0.08  | 6.49 ± 0.15     |
| 3    | 30 (-1)                | 60 (-1)    | 80                          | 3.23 ± 0.23    | 1.67 ± 0.04   | 18.90 ± 0.16  | 4.13 ± 0.05     |
| 4    | 30 (-1)                | 180 (+1)   | 20 (-1)                     | 5.98 ± 0.38    | 2.30 ± 0.05   | 31.55 ± 0.20  | 5.41 ± 0.07     |
| 5    | 30 (-1)                | 180 (+1)   | 80                          | 2.58 ± 0.10    | 1.90 ± 0.06   | 23.86 ± 0.20  | 5.59 ± 0.27     |
| 6    | 45 (0)                 | 31.7 (-1.47)| 50 (0)                     | 7.38 ± 0.24    | 2.70 ± 0.16   | 30.20 ± 0.24  | 7.41 ± 0.26     |
| 7    | 45 (0)                 | 120 (0)    | 5.9 (-1.47)                | 7.01 ± 0.55    | 1.77 ± 0.09   | 26.97 ± 0.28  | 5.79 ± 0.03     |
| 8    | 45 (0)                 | 120 (0)    | 50 (0)                      | 7.24 ± 0.44    | 2.82 ± 0.09   | 31.35 ± 0.25  | 8.97 ± 0.58     |
| 9    | 45 (0)                 | 120 (0)    | 94.1 (+1.47)               | 3.39 ± 0.17    | 1.36 ± 0.04   | 19.43 ± 0.42  | 6.36 ± 0.08     |
| 10   | 45 (0)                 | 208.3 (+1.47)| 50 (0)                  | 5.65 ± 0.25    | 3.04 ± 0.12   | 30.42 ± 0.04  | 7.62 ± 0.08     |
| 11   | 60 (+1)                | 60 (-1)    | 20 (-1)                     | 6.39 ± 0.21    | 2.58 ± 0.12   | 32.58 ± 0.08  | 5.99 ± 0.07     |
| 12   | 60 (+1)                | 60 (-1)    | 80                          | 5.43 ± 0.46    | 2.27 ± 0.14   | 29.62 ± 0.43  | 6.68 ± 0.03     |
| 13   | 60 (+1)                | 180 (+1)   | 20 (-1)                     | 5.24 ± 0.36    | 2.88 ± 0.10   | 28.67 ± 0.23  | 4.69 ± 0.06     |
| 14   | 60 (+1)                | 180 (+1)   | 80                          | 4.55 ± 0.14    | 2.55 ± 0.13   | 28.20 ± 0.30  | 8.46 ± 0.15     |
| 15   | 67.1 (+1.47)           | 120 (0)    | 50 (0)                      | 5.90 ± 0.59    | 3.28 ± 0.22   | 36.17 ± 0.23  | 8.47 ± 0.13     |
|      | Total                  |            |                             | 5.76 ± 1.54    | 2.47 ± 0.51   | 29.14 ± 4.21  | 7.09 ± 1.59     |

All values are mean ± SD of the three replicates; TPC, total polyphenol content; TFC, total flavonoid content; GAE, gallic acid equivalent; QE, quercetin equivalent; TEAC, Trolox equivalent antioxidant capacity; DPPH, 2,2'-diphenyl-1-pikrylhydrazyl; TE, Trolox equivalent.
aliquot of 20 µl of BS extract was mixed with 100 µl of FCR and left in the dark for 3 min (at ambient temperature). Then, 300 µl of 20% Na₂CO₃ was added, and filled with distilled water up to a volume of 2 ml. The sample was incubated in a dark place for 2 h at room temperature. Absorbance readings were taken at 765 nm against a blank sample (prepared in the same manner with 20 µl of extraction solvent instead of BS extract). The results were expressed as gallic acid equivalent (GAE) in mg per g of BS.

TFC (total flavonoid content)

TFC was assessed by means of the aluminum chloride method [23] as described previously [19]. Briefly, 100 µl of BS extract was mixed with 900 µl of 2% aluminum chloride in methanol and the absorbance was read at 415 nm, after 15 min of incubation in the dark at ambient temperature. Pure methanol was used as a blank sample. The results were expressed in mg of quercetin equivalent (QE) per g of BS.

TEAC assay

The radical scavenging capacity of extracts was assessed by the TEAC (Trolox Equivalent Antioxidant Capacity) assay [24] with some modifications [25]. The ABTS⁺⁺ radical cation was generated by mixing 7 mM ABTS in water with 2.45 mM potassium disulphate at a ratio of 2:1. To produce radicals the mixture was left in the dark at ambient temperature for 12–16 h. Then, the ABTS⁺⁺ solution was diluted with PBS (phosphate buffer saline), pH 7.4 to an absorbance of 0.7 (± 0.01). Next, 990 µL of ABTS⁺⁺ solution was mixed with 10 µL of BS extract and, after 6 min of incubation in the dark (ambient temperature), the decrease in the absorbance of the mixture was measured on a Cary 1E spectrophotometer (Varian, Belrose, Australia) at 734 nm against the blank sample (ABTS⁺⁺ solution with solvent). The results were expressed as the TEAC values in micromolar concentrations (µmol) per g of BS.

DPPH radical scavenging capacity

The DPPH⁺ free radical-scavenging activity measurements were carried out spectrophotometrically at 515 nm according to the method presented by Sánchez-Moreno et al. [26]. Briefly, stable DPPH⁺ radicals (0.1 mmol) were generated in methanol. Then, an aliquot of 10 µl of BS extract was added to 990 µl of DPPH⁺ in methanol and mixed. The absorbance was read after 30 min of sample incubation in the dark using pure methanol as a reference. The DPPH⁺ radical scavenging activity of BS extract was expressed as a Trolox equivalent (TE) (µmol/g) calculated from the ratio of the slope of the linear plot for scavenging DPPH⁺ radicals by BS extract to the slope of the plot for DPPH⁺ radicals scavenging by the Trolox.

HPLC (high performance liquid chromatography) measurements

The composition and content of phenolic compounds in BS extracts were determined using the HPLC method developed by Enko and Gliszczyńska-Świgło [27]. In this study, gallic, protocatechuic, p-hydroxybenzoic, chlorogenic and caffeic acids, as well as rutin, quercetin, apigenin, naringenin, diosmin, free quercetin and kaempferol were tested in the extracts. Quercetin and kaempferol derivatives were found and identified based on their absorption spectra, their stability after alkaline hydrolysis and their decline under acid hydrolysis, and identification of quercetin and kaempferol as the main flavonoids in the solution after alkaline-acid hydrolysis. The quantification of quercetin and kaempferol derivatives was conducted at 365 nm using the external standard method with kaempferol and quercetin as standards. The results were expressed in µg quercetin or kaempferol/g seeds. Alkaline and acid hydrolysis was performed as described previously [28]. Each sample analysis was run in triplicate.

Statistical analysis

Statistical analysis was performed using Statistica 13.3 software. All results were expressed as means with standard deviations. The adequacy of the second-order polynomial model Eq. (1) was evaluated by the coefficient of determination (R²), adjusted coefficient of determination (adj. R²), CV (coefficient of variance, %), lack of fit, and the p-value (F-test) obtained for the analysis of variance (Anova). The correlation coefficients between the experimental and predicted values were calculated to validate the model. The single- and multi-response optimization for maximizing the phenolic content and the radical scavenging activity was obtained using desirability functions. Multilinear regression analysis was used to assess the effect of the independent variable on the content of quercetin and kaempferol derivatives. BETA coefficients were calculated to show the importance of the independent variables for the response variable. All analyses were performed with a 95% confidential level.
Table 2: ANOVA of the models fitted to phenolic content and the antioxidant activity of black seed (*Nigella sativa*) extracts

| Source          | TPC (mg GAE/g) SS | TPC (mg GAE/g) p | TFC (mg QE/g) SS | TFC (mg QE/g) p | TEAC (µmol/g) SS | TEAC (µmol/g) p | DPPH (µmol TE/g) SS | DPPH (µmol TE/g) p |
|-----------------|------------------|-----------------|-----------------|----------------|-----------------|----------------|----------------------|--------------------|
| T               | 8.59             | 0.00            | 3.09            | 0.00           | 131.36          | 0.00           | 11.12                | 0.00               |
| T²              | 28.16            | 0.00            | 0.01            | 0.28           | 22.14           | 0.00           | 15.90                | 0.00               |
| t               | 7.60             | 0.00            | 0.61            | 0.00           | 0.66            | 0.00           | 0.33                 | 0.11               |
| t²              | 3.10             | 0.00            | 0.01            | 0.40           | 7.57            | 0.00           | 17.72                | 0.00               |
| c               | 44.18            | 0.00            | 0.99            | 0.00           | 267.46          | 0.00           | 2.35                 | 0.00               |
| c²              | 24.74            | 0.00            | 9.64            | 0.00           | 406.51          | 0.00           | 59.78                | 0.00               |
| Tt              | 0.39             | 0.11            | 0.00            | 0.65           | 53.87           | 0.00           | 0.00                 | 0.87               |
| Tc              | 8.89             | 0.00            | 0.01            | 0.44           | 86.76           | 0.00           | 16.53                | 0.00               |
| tc              | 0.00             | 0.98            | 0.00            | 0.72           | 12.40           | 0.00           | 11.83                | 0.00               |
| Lack-of-fit     | 1.01             | 0.242           | 0.02            | 0.867          | 0.74            | 0.052          | 1.24                 | 0.096              |
| pure error      | 6.03             | 0.46            |                 |                |                 |                | 5.14                 |                    |
| Model           | 125.67           | 0.00            | 14.36           | 0.00           | 988.73          | 0.00           | 135.59               | 0.00               |
| Residual        | 7.04             |                 | 0.48            |                 | 3.33            | 6.38           |                      |                    |
| Total           | 132.71           |                 | 14.84           |                 | 992.06          | 141.97         |                      |                    |
| R²              | 0.947            |                 | 0.968           |                 | 0.997           | 0.955          |                      |                    |
| adj. R²         | 0.937            |                 | 0.961           |                 | 0.996           | 0.946          |                      |                    |
| CV (%)          | 6.21             | 3.79            | 0.84            |                 | 4.8             |                |                      |                    |

∀, temperature; t, time; c, methanol concentration; adj R², adjusted R²; CV, coefficient of variance; TPC, total polyphenol content; TFC, total flavonoid content; GAE, gallic acid equivalent; QE, quercetin equivalent; TEAC, Trolox equivalent antioxidant capacity; DPPH, 2,2’-diphenyl-1-pikrylhydrazyl; TE, Trolox equivalent; significant at p ≤ 0.05

Results and discussion

Fitting the models

To find optimal extraction conditions (time, temperature and methanol concentration) in terms of finding the maximal response values of TPC, TFC and the radical scavenging capacity (DPPH’ and ABTS⁺) RSM using CCP (central composite plan) based on common factorial 2³ design with star and center points added was employed. The total number of replicates at the center point was 15 in order to determine the lack-of-fit and pure error parameters used for evaluating model accuracy. To avoid possible bias errors, the samples were randomized. The experimental results together with coded and uncoded values of independent variables are shown in Table 1.

Anova was used for testing the models’ accuracy and the model suitability was confirmed by the F-test (Table 2). The F statistics for the model were high and the corresponding p values were below 0.05 (p = 0). The model quality was also verified by the lack-of-fit (as a component of residual error) for all responses, since the replication in the central points were included. The parameter was insignificant (p > 0.05), which means that the residual SS (sum of squares) was not significantly higher than the pure error SS (Table 2), thus the linear/quadratic relationship with linear interactions assumed in the model was reasonable. All determination coefficients R² and adj. R² were high and indicated that around 95% of the variability for TPC, TFC and DPPH responses and above 99% of the variability for TEAC response were explained by the models built. Furthermore, CV values, which represent the relative dispersion of data in a data set around the mean and enable comparison of the degree of variation between data sets expressed in various units, were below 10% indicating good reproducibility of the studies. Experimental data was used for building the three-dimensional response surface for each dependent variable (Fig. 1). All surface plots were generated by keeping one independent variable at a constant level (corresponding to the center point value) while the values of other two variables (shown on the axes) varied in the range being studied.

Analysis of response surface for phenolic content (TPC and TFC)

Based on the results of the Anova, it could be stated that all independent variables: time, temperature and methanol concentration significantly influenced the TPC values. The effects of the linear (t, T, c), quadratic (T², T, c²) of the three factors (time, temperature, methanol concentration) and linear interaction of temperature and methanol concentration (T × c) determined by the response surface analysis showed p values < 0.05 (Table 2). All other effects were statistically insignificant. Moreover, the linear effect of methanol concentration (c), quadratic effect of temperature (T²) and quadratic effect of methanol (c²) were the most important in the model built with the standardized effect absolute values...
Positive values for the regression coefficients indicated that the terms of the model increased the response whereas negative ones decreased it. All linear components showed positive effects for the extraction of polyphenolic compounds from BS, whereas quadratic components of the model with negative regression coefficients tended to decrease the TPC. However, the quadratic function employed suggested that TPC values increased with the increased values of the independent variables and after reaching a saddle point decreased, which was illustrated by the three-dimensional response surface plot for TPC (Fig. 1).

TPC increased along with an increase in temperature, methanol concentration and time up to 45ºC, 28%

\[ TP_C = -17.5 + 0.382 \times T - 0.0045 \times T^2 + 0.021 \times t - 0.00009 \times t^2 + 0.0071 \times c - 0.001 \times c^2 - 0.00014 \times T \times t + 0.0014 \times T \times c - 0.000001 \times t \times c \]
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Table 3 Predicted and observed values of all responses (TPC, TFC, TEAC and DPPH) under optimal conditions calculated from single- and multi-response desirability functions

| Variables         | Temperature (°C) | time (min) | methanol concentration (%) | desirability function coefficient | predicted | LPI      | UPI      | validation |
|-------------------|-----------------|------------|----------------------------|-----------------------------------|-----------|----------|----------|------------|
| single response   |                 |            |                            |                                   |           |          |          |            |
| TPC (mg GAE/g)    | 45              | 76         | 28                         | 0.92                              | 7.68      | 8.49     | 6.78 ± 0.33 |            |
| TFC (mg QE/g)     | 67              | 208        | 50                         | 1                                 | 3.54      | 3.79     | 3.15 ± 0.03 |            |
| TEAC (μmol/g)     | 67              | 76         | 50                         | 1                                 | 37.31     | 37.9     | 36.83 ± 1.40 |            |
| DPPH (μmol TE/g)  | 45              | 120        | 50                         | 0.925                             | 9.04      | 9.8      | 12.56 ± 0.57 |            |
| multi-response    |                 |            |                            |                                   |           |          |          |            |
| TPC (mg GAE/g)    | 56              | 120        | 50                         | 0.831                             | 7.05      | 7.85     | 6.48 ± 0.15 |            |
| TFC (mg QE/g)     |                  |            |                            |                                   | 3.05      | 3.25     | 3.10 ± 0.04 |            |
| TEAC (μmol/g)     |                  |            |                            |                                   | 33.24     | 33.79    | 33.22 ± 0.32 |            |
| DPPH (μmol TE/g)  |                  |            |                            |                                   | 9.04      | 8.27     | 9.8 ± 1.35  |            |

all values are mean ± SD of the three replicates; LPI, lower predictive interval; UPI, upper predictive interval; TFC, total flavonoid content; TFC, total flavonoid content; GAE, gallic acid equivalent; QE, quercetin equivalent; TEAC, Trolox equivalent antioxidant capacity; DPPH, 2,2'-diphenyl-1-pikrylhydrazyl; TE, Trolox equivalent

and 76 min, respectively, reaching the maximal predicted value of 7.68 mg GAE/g (Table 3). Following that, a sharp decrease in TPC with a further increase in temperature up to 67°C and methanol concentration up to 94% was observed (at time = 120 min). A consecutive increase in extraction time from 76 to 208 min resulted in a slight decrease in TPC values (Fig. 1).

Anova performed for the TFC response confirmed the significance of all linear effects of independent variables (*t, T, c*), and the quadratic effect of methanol concentration (*c*²), the latter with the highest impact on the TFC based on the standardized effect estimate. Although all linear interactions between independent variables for TFC response were statistically insignificant, excluding these interactions from the model resulted in only a slight, statistically insignificant increase in R² and adj. R², whereas the increase in the p value for lack-of-fit was from 0.87 up to 0.94. Thus, the multiple regression analysis of the experimental data enabled to obtain the second-order polynomial regression equation as given:

\[
\begin{align*}
TFC & = 0.03 + 0.0005 \times T + 0.0008 \times c + 0.0002 \times c^2 + 0.0005 \times t + 0.0056 \times c - 0.0002 \times t^2 \\
\end{align*}
\]  

(3)

and after excluding the interaction terms as followed:

\[
\begin{align*}
TFC & = 0.07 + 0.0006 \times T + 0.0009 \times T^2 + 0.0002 \times t + 0.0005 \times t^2 - 0.0056 \times c + 0.0006 \times c^2 \\
\end{align*}
\]  

(4)

As shown from response surface (Fig. 1), TFC increased with an increase in extraction time and temperature up to the highest levels of these factors (within the range studied), whereas an increase in TFC with an increase of methanol concentration up to the concentration of 50% and then a decrease with consecutive increase in methanol concentration was observed. Therefore, the maximal value of TFC was predicted as 3.54 mg QE/g at *T* = 67°C, *t* = 208 min and *c* = 50% (Table 3).

The effects of temperature and solvent concentration were crucial for the extraction of phenolic compounds from plant material. The rise in temperature could have a positive effect on the solubility of the compounds and diffusion coefficient [32] and thus increased the content of phenolics in the extract, as was noticed for the TFC response. However, an intense increase in temperature could have caused degradation of phenolic compounds by various redox reactions and polymerization [33] and finally lowered the level of extracted polyphenols, which was observed in this study for TPC values. The effect of solvent concentration on the content of phenolic compounds could be explained based on the polarity of the solvent used for the extraction. Phenolic compounds have a high number of hydroxyl groups in their structure and are often present in plant tissues as glycosides. This results in the high diversity of these compounds and their properties like solubility. Although pure alcohol used as a solvent for phenolic extraction could cause degradation of lipid cell membranes and thus make the release of the compounds from plant cells easier [34], its polarity could be too low for the recovery of the most phenolics, as was observed in this study. Generally, increasing the polarity of the alcohol by mixing it with water facilitated breakage of hydrogen bonds in polyphenols structure and improved their solubility in organic solvents [17]. Binary water-alcohol solvent was shown to increase the efficiency of the extraction process in comparison to pure solvent [28, 30, 35, 36].

Although some researchers reported that prolonged extraction time, especially at high temperature, led to a decrease in the phenolic content of the extract as oxidation...
and further degradation of the compounds could occur [37, 38], keeping the sample in the solvent at a constant, relatively low temperature (up to 60°C) for the maximum extraction time allowed the compounds to migrate to the solvent from the plant material [37]. In this study, the final effect of time/temperature factors on the content of phenolic compounds was varied when comparing TPC and TFC responses, which was probably related to the phenolic profile of the extracts.

Previously, TPC and TFC of BS had been determined by other authors in different extraction systems (time/temperature, solvent type and concentration – data collected in Table 4) [2, 3, 11, 15, 39, 40], and expressed in various units, thus it is hard to compare our data with studies in the literature. However, a significant effect of solvent concentration and the influence of extraction time could be seen from literature data (Table 4). The level of TPC in the extract of BS seeds varied from 0.63 mg GAE/g for the 100% ethanol extract [15] to 2.93 mg GAE/g for the 70% methanol extract (unknown extraction time) [2]. For pure ethanol, the extraction of TPC value was lower than for aqueous ethanol after 30 min or 2 h extraction [11, 15]. Similar tendency was observed for methanol solvent. Samples prepared in pure methanol had lower TPC than samples prepared using aqueous methanol [2, 15]. However, when extending time of extraction to 24 h, TPC was higher in pure ethanol than in 50% aqueous alcohol [11]. Surprisingly, values of TPC obtained for the samples extracted with pure water were not the lowest and varied from 1.80 mg GAE/g after 24 h extraction to 2.63 mg GAE/g (unknown extraction time) [2, 11].

### Analysis of response surface for the radical scavenging capacity

RSM was also successfully applied for optimization of the conditions to maximize the radical scavenging capacity of the extracts using the TEAC and DPPH methods (Table 2; Fig. 1). It could be concluded, based on the Anova performed for the TEAC values, that all effects, namely the linear, quadratic and linear interactions of the factors: time, temperature and methanol concentration were statistically significant ($p \leq 0.05$). Methanol concentration ($c^2$ and $c$) showed the highest impact on the response, according to the absolute values of standardized effects (-81.2 and -65.9, respectively), whereas time showed the lowest (standardized values -11.1 and 3.3 for $t^2$ and $t$, respectively). The response surface equation was as followed:

$$TEAC=A 	imes T 	imes c^2 - B 	imes T^2 + C 	imes c + D,$$  

where $T$ is temperature, $c$ is methanol concentration, $A$, $B$, $C$, and $D$ are coefficients for the linear, quadratic term of temperature ($T$), quadratic term of time ($t^2$), linear term of methanol concentration ($c^2$) and linear time-temperature interaction ($T \times t$) in the equation. For pure methanol $c=1$ and $T=0$, for pure water $c=0$ and $T=1$. For the 70% methanol extract, $c=0.7$ and $T=0.3$. The response surface equation was calculated for TEAC values of 24.89 mg GAE/g (Table 2).

### Table 4: Maximum total polyphenol content (TPC) and total flavonoid content (TFC) of black seeds at various extraction conditions – literature data

| Extraction conditions (solvent, time, temperature) | TPC (mg GAE/g) | TFC (mg QE/g) | Source |
|---------------------------------------------------|----------------|---------------|--------|
| water, unknown time, ambient temperature          | 2.63           | 1.68          | [2]    |
| 70% aqueous methanol, unknown time, ambient       | 2.93           | 1.74          | [2]    |
| temperature                                      |                |               |        |
| 30% aqueous ethanol, 12 h, 4 °C                   | 2.79           | 0.41          | [3]    |
| 70% aqueous ethanol, Soxhlet apparatus at         | 35.72*         | -             | [13]   |
| boiling point of the solvent, 15 cycles           |                |               |        |
| water, Soxhlet apparatus at boiling               | 24.89*         | -             | [13]   |
| point of the solvent, 15 cycles                   |                |               |        |
| 100% methanol, 30 min at ambient temperature      | 11.69*         | -             | [39]   |
| + 24 h, 4 °C                                       |                |               |        |
| 100% ethanol, 24 h, ambient temperature           | 2.61           | 0.59          | [11]   |
| 100% ethanol, 30 min, ambient temperature         | 0.95           | 0.47          | [11]   |
| 50% aqueous ethanol, 24 h, ambient temperature    | 2.46           | 0.98          | [11]   |
| 50% aqueous ethanol, 30 min, ambient temperature | 1.51           | 0.77          | [11]   |
| water, 24 h, ambient temperature                  | 1.80           | 1.61          | [11]   |
| water, 3 h, ambient temperature                   | 1.81           | 1.04          | [11]   |
| 80% aqueous methanol, 28 h, ambient temperature   | 4.43**         | -             | [40]   |
| methanol, 2 h, ambient temperature (from defatted) | 0.92***        | -             | [15]   |
| 70% methanol, 2 h, ambient temperature (from      | 1.14***        | -             | [15]   |
| defatted seeds)                                   |                |               |        |
| ethanol, 2 h, ambient temperature (from defatted) | 0.63***        | -             | [15]   |
| 70% ethanol, 2 h, ambient temperature (from       | 1.03***        | -             | [15]   |
| defatted seeds)                                   |                |               |        |
| 50% aqueous methanol, 120 min, 56 °C              | 6.48           | 3.10          | This   |
| study                                             |                |               |        |

*in mg GAE per g of dry matter, lack of information on yield of extraction; ** in methodology description TPC was expressed in mg GAE per g of powdered extract, lack of data on extraction yield; *** calculated from mg GAE per g of dry matter using data on extraction yield.
a temperature of 23°C. The radical scavenging capacity increased with the increase in methanol concentration, reaching the maximal TEAC values with 50% methanol and a further increase in methanol concentration decreased the response. Thus, the maximal TEAC values were obtained at: 67°C, 76 min and 50% methanol (Table 3). In the previous study, it was reported that the ABTS+ radical scavenging capacity of BS extract obtained with 70% methanol was significantly higher than for the extract obtained with water and stood at 67.2 µmol TE/g and 53.6 µmol TE/g, respectively [2]. The values reported by Hameed et al. [2] were around twice as high as those predicted from the equation in this study.

With respect to the radical scavenging capacity investigated by means of the DPPH method, linear and quadratic effects of temperature (T and T²), and methanol concentration (c and c²), linear effect of time (t), and two of three interactions (T x c and t x c) were statistically significant (p ≤ 0.05) (Table 2). As for TFC or TEAC responses, the quadratic term of methanol concentration (c²) was the most important in the model fitted to DPPH data, which was concluded based on the absolute value of standardized effect equaled to -22.1. The second-order polynomial model for DPPH data was as given:

\[
D_{PPH} = 6535 + 0.0344 \times c + 0.000221 \times c^2 - 0.00039 \times T + 0.001621 \times t - 0.000013 \times (T \times c) - 0.0003344 \times (T \times t) + 0.000013 \times (c \times t) + 0.000013 \times (t \times c) - 0.000013 \times (c \times c) - 0.000013 \times (T \times T) - 0.000013 \times (t \times t)
\]

It was shown that all regression coefficients for quadratic terms in the model built had negative values, indicating that the DPPH values increased when temperature, time and solvent concentration increased up to 45°C, 120 min and 50%, respectively, and after reaching the maximal value of 9.04 µmol TE/g, dropped sharply with a further increase of the factors (Fig. 1). A similar influence of solvent concentration on the antioxidant activity of plant was previously observed by other authors [30, 31, 41]. Generally, solvent concentration was a crucial parameter for the extraction of phenolics and other antioxidants from plant materials and it was noticed that the alcohol concentration in water for maximizing the antioxidant activity of plants varied from 45 to 60%, which is in accordance with our studies.

**Single- and multi- response optimization and validation of the models**

The desirability functions were used for the optimization procedures conducted with the RSM models in order to maximize the individual response variables. The optimal conditions of temperature, time and solvent concentration for single responses are shown in Table 3. TPC required a lower methanol concentration (28%), shorter time (76 min) and lower temperature (45 °C) for the maximal concentration than TFC (50%, 208 min, 67 °C). The maximal radical scavenging capacity measured by the TEAC and DPPH methods was obtained after extraction with 50% aqueous methanol. However, the DPPH value was maximized at a lower temperature (45 °C) and after longer extraction (120 min) than the TEAC value (67 °C, 76 min, respectively). Desirability function coefficients calculated from the single-response functions were 0.92 for the TPC response, 1.00 for TFC and TEAC responses and 0.93 for the DPPH response, which means that 92%, 100% and 93% of the proposed aims, respectively, were found by the optimization procedure. To verify the models and therefore the optimal conditions, an external validation was performed. Table 3 includes the predicted and experimentally obtained (validation) values for single-responses in optimal conditions. The observed TPC, TFC and the TEAC values (6.78 mg GAE/g, 3.15 mg QE/g and 36.83 µmol/g, respectively) were in the predictive intervals (95%) or close to their values, whereas the experimentally obtained DPPH value (12.56 µmol TE/g) was higher than the predicted one (9.04 µmol TE/g) and higher than the expected 95% upper predictive interval (UPI). However, since the goal of the RSM was to maximize the antioxidant activity, a higher value than the predicted one was acceptable. Different response variables required different optimal conditions to maximize them (Table 3; Fig. 1). Hence, there is a need to determine the extraction conditions, which would simultaneously provide the highest phenolic content and the antioxidant activity. This was performed by employing multi-response optimization. The optimal conditions for maximizing all responses at once were as followed: a temperature of 56 °C, a time of 120 min and a methanol concentration of 50% (Table 3). At this optimal point, the predicted TPC was 7.05 mg GAE/g, predicted TFC was 3.05 mg QE/g, the predicted TEAC value was 33.24 µmol/g and the predicted DPPH value was 9.04 µmol TE/g. The predicted values of TPC, TFC and TEAC match well to the experimental results obtained under the optimal conditions for the multi-response function (Table 3). The DPPH value measured was significantly higher than the predicted one, just as was observed in single-response optimization. Generally, satisfactory agreement between predicted and observed data at optimal conditions for single- and multi-response functions proved the suitability and high accuracy of the RSM models for quantitative prediction of the phenolic content and the antioxidant activity of the BS extract (r Pearson’s correlation coefficient of 0.99). The most desirable combinations were further analyzed for the phenolic profile.
Phenolic profiles in the optimized extraction conditions

BS extracts prepared in the optimal conditions (from Table 3) were investigated by HPLC in order to identify the individual phenolic compounds responsible for the radical scavenging activity of the extracts. Three phenolic compounds were identified in *Nigella sativa* extracts, which were the quercetin and kaempferol derivatives (Table 5; Fig. 2) with kaempferol derivatives being the most predominant flavonoids in the methanolic BS extracts. When comparing the elution order of the compounds determined with the literature data [12, 13], the quercetin derivative was tentatively identified as quercetin-O-α-rhamnosyl-triglucoside or quercetin-3-O-sophoroside-7-glucoside, whereas kaempferol derivatives would be kaempferol-3-O-sophorotrioside-7-O-rhamnoside and kaempferol-3-O-[β-D-glucopyranosyl-(1–2)-β-D-galactopyranosyl-(1–2)-β-D-glucopyranoside], the latter being the most abundant in the extracts. Similar results were obtained by Toma et al. [14] and Zwolan et al. [13], who reported the presence of kaempferol derivatives as the most abundant phenolic compounds in a 70% ethanolic extract of *Nigella sativa*, and by Topcagic et al. [1], who reported kaempferol glycosides as the predominant compounds in a 80% methanolic extract. In this study, other phenolic compounds such as gallic, protocatechuic, p-hydroxybenzoic, chlorogenic and caffeic acids, as well as rutin, quercetin, apigenin, naringenin, diosmin, free quercetin and kaempferol were not found in the extracts tested, although others have been reported previously by Zwolan et al. [13].

As could be concluded from regression analysis of the results from Table 5, the extraction conditions - methanol concentration and temperature - were shown to affect the content of all 3 compounds significantly. Time was statistically insignificant. The highest content of quercetin and kaempferol derivatives was obtained under the optimal conditions calculated for TEAC and TFC single-responses.

Conclusion

RSM with CCP was applied for the optimization of extraction conditions, namely, time, temperature and methanol concentration in order to maximize the TPC, TFC and the radical scavenging activity (ABTS** and DPPH**) of *Nigella sativa* extracts. All RSM models were well fitted to the experimental data with low CV values (0.84–6.21%) indicating good reproducibility of the data, and high $R^2$ (0.95–0.997) indicating that more than 95% of the variability was explained by the models built. The effect of solvent concentration was crucial for the extraction of phenolics and other antioxidants from *Nigella sativa* seeds. Based on single- and multi-response desirability functions, the optimal conditions were determined with the high desirability function coefficients (0.83–1). The antioxidant potential and total polyphenols in *Nigella sativa* can be brought to the maximal level by different combinations of methanol concentration, time and temperature. Based on the multi-response surface model, the optimal conditions for all responses (TPC, TFC, the antioxidant activity) were as followed: temperature of 56 °C, time of 120 min and methanol concentration of 50% which was similar to the optimal conditions calculated using single-response surface methodology for DPPH values. External validation proved the high accuracy of the RSM models for quantitative prediction of the phenolic content and the antioxidant activity of *Nigella sativa* extract (correlation coefficient equals to 0.99). In all extracts, one quercetin derivative and two kaempferol derivatives were identified, showing the importance of these compounds in the antioxidant activity of the *Nigella sativa* extract. Concluding, extract of *Nigella sativa* seeds could be a good source of bioactive compounds, which could be further used as natural antioxidants in the industry. However, the extraction conditions significantly affected the final responses, thus optimization procedure is a useful tool for providing extracts with both a high phenolic content and high radical scavenging activity.

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| ABTS | 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) |
| BS | black seed |
| CCP | central composite plan |
| CV | coefficient of variance |
| DPPH | 2,2’-diphenyl-1-picrylhydrazyl |
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Fig. 2 HPLC chromatograms of the extract obtained under the optimal conditions for multi-response optimization: (a) before hydrolysis, (b) after alkaline-acid hydrolysis: Q – quercetin, K – kaempferol

| Acronym | Description                                      |
|---------|--------------------------------------------------|
| FCR     | Folin-Ciocalteu phenol reagent                   |
| GAE     | gallic acid equivalent                           |
| HPLC    | high performance liquid chromatography           |
| K       | kaempferol                                       |
| LPI     | lower predictive interval                        |
| Q       | quercetin                                        |
| QE      | quercetin equivalent                             |
| RSM     | response surface methodology                     |
| SS      | sum of squares                                   |
| TEAC    | Trolox Equivalent Antioxidant Capacity           |
| TFC     | total flavonoid content                          |
| TPC     | total polyphenol content                         |

Note: K represents kaempferol, and Q represents quercetin.
CRediT authorship contribution statement
Malgorzata Muzolf-Panek: Conceptualization, resources, investigation, methodology, data curation, formal analysis, writing – original draft, writing – review and editing, visualization, project administration; Anna Gliszczyńska-Świgło: HPLC methodology and investigation, writing – review and editing.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Compliance with ethics requirements
This publication does not contain any human or animal experimental data and features only experiments without human participants or animal subjects.

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Table 5: Phenolic compounds (µg/mL) of black seed (Nigella sativa) extracts prepared in optimal conditions derived from single- and multi-response functions

| Compound concentration µg/g seed | Quercetin | Kaempferol derivative 1 kaempferol-3-O-[β-D-glucopyranosyl(1–2)-β-D-glucopyranosyl]-7-O-rhamnoside | Kaempferol derivative 2 kaempferol-3-O-[β-D-glucopyranosyl(1–2)-β-D-glucopyranosyl]-7-O-rhamnoside | Total flavonol derivatives |
|---------------------------------|-----------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|--------------------------|
| Temperature* (°C)               | 45   | 19.80 ± 0.30 | 198.05 ± 0.04 | 67.90 ± 0.05 | 364.80 ± 0.10 |
| time (min)                      | 67   | 20.25 ± 0.40 | 202.50 ± 0.40 | 67.90 ± 0.05 | 364.80 ± 0.10 |
| methanol concentration (%)      | 76   | 20.78 ± 0.15 | 207.80 ± 0.15 | 67.90 ± 0.05 | 364.80 ± 0.10 |
| Quercetin derivative 2          | 67   | 20.18 ± 0.38 | 201.80 ± 0.38 | 67.90 ± 0.05 | 364.80 ± 0.10 |
| methanol concentration (%)      | 76   | 20.78 ± 0.15 | 207.80 ± 0.15 | 67.90 ± 0.05 | 364.80 ± 0.10 |
| Total flavonol derivatives      | 67   | 20.18 ± 0.38 | 201.80 ± 0.38 | 67.90 ± 0.05 | 364.80 ± 0.10 |

* significant effect (calculated from regression analysis, p ≤ 0.05), † based on the elution order [12, 13], the absorption spectra and the decline after acid hydrolysis

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