

OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF POLYPHENOLS FROM HONEYSUCKLE (*Lonicera caprifolium*)

Luminița DIMITRIU^{1,2}, Daniel PREDA¹, Diana CONSTANTINESCU-ARUXANDEI¹,
Florin OANCEA¹, Narcisa BĂBEANU²

¹National Institute for Research & Development in Chemistry and Petrochemistry - ICECHIM, 202
Splaiul Independenței, Bucharest, Romania

²University of Agronomic Sciences And Veterinary Medicine of Bucharest, 59 Mărăști Blvd,
Bucharest, Romania

Corresponding author email: diana.constantinescu@icechim.ro

Abstract

Honeysuckle (*Lonicera caprifolium*) is a perennial flowering plant from the genus *Lonicera* of the family Caprifoliaceae. *Lonicera caprifolium* flowers are used in herbal traditional medicine for their antibacterial, antioxidant and antiviral activities. The objective of this research was to optimize the ultrasound-assisted extraction (UAE) process of polyphenols from *Lonicera caprifolium* flowers using Design of Experiments (DoE) and Response Surface Methodology (RSM). We used a three-level factorial model with three variable factors (ethanol concentration, time and the ratio between solvent and plant material). The model and the data indicated that the ethanol concentration was highly significant ($p < 0.05$), while the other two were less significant for the extraction of the maximum total phenolic compounds and the maximum antioxidant activity of the extracts. The antioxidant activity was performed by DPPH, ABTS and FRAP methods. The polyphenols were identified by HPLC. The optimal condition obtained for the extraction of polyphenols from honeysuckle by UAE will be used for bioproduct formulation in future work.

Key words: antioxidant activity, polyphenols, response surface methodology, ultrasound-assisted extraction.

INTRODUCTION

Polyphenols are secondary metabolites of plant material with various biological properties such as antioxidant, antimicrobial, anti-inflammatory, anti-hypertensive and other properties (Gam et al., 2020). The main classes of polyphenols include phenolic acids, flavonoids, stilbenes and lignans (Abbas et al., 2017).

Due to their important biological activity we have recently seen an increased interest in research about their extraction, isolation, identification and activity. Numerous studies show relationships between the polyphenol content and the antioxidant activity of plant material (Proteggente et al., 2002; Nichita et al., 2016; Rodriguez-Casado et al., 2016). The method of extraction of phenolic compounds influences their bioavailability, their content, and their quality. For this reason new methods of extraction of polyphenols were investigated and developed lately (Chemat et al., 2017).

Ultrasound-assisted extraction (UAE) is described as green, efficient, easy to operate

method. UAE is based on the phenomenon of acoustic cavitations and mechanical effects on plant matrix, which can lead to the disintegration of the cell wall and allows the rapid passing of the solvent through the plant matrix and consecutive intensification of the extraction process (Ali et al., 2018).

Lonicera caprifolium is an European perennial flowering plant from the genus *Lonicera* of the family Caprifoliaceae. *L. caprifolium* flowers are used in herbal traditional medicine for their antibacterial, antioxidant and antiviral activities (Ilies et al., 2013). While many species of genus *Lonicera* such as *L. japonica* (Japanese honeysuckle) and others are studied and used in traditional medicine and cosmetics, the chemical composition of European honeysuckle (*L. caprifolium*) is less investigated (Seo et al., 2012).

The main aim of this research was to determine the optimal conditions for the hydroalcoholic UAE of polyphenols from local *L. caprifolium* and to establish the polyphenolic composition of the extract resulted in optimal conditions. The extraction was monitored in terms of

optimal content of polyphenols/compounds reacting with Folin-Ciocalteu reagent and optimal antioxidant activity.

MATERIALS AND METHODS

Materials

Freshly flowers were harvested from the plants of *L. caprifolium* in Bucharest. The flowers were dried at room temperature and were ground to a powdered form using an electrical grinder. The following chemicals were used: pharmaceutical ethanol 96% (Chimopar Srl, Bucharest, Romania), Trolox 97% (Acros Organics, Thermo Fisher Scientific, Pittsburghs, PA, USA), Gallic acid, 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Merck Group, Darmstad, Germany), 2,2'-Azino-bis (3-ethylbenzothiazole-6-sulfonic acid) diammonium salt, 98%, 2,4,6-tri (2-piridil-1,3,5-triazina) 98% (Alfa Aesar), Folin-Ciocalteu's phenol reagent, Iron chloride (III) (Merk), hydrochloric acid, acetic acid (Chimopar, Bucharest), sodium acetate (Scharlau, Barcelona, Spain), HPLC standards: ferulic acid, p-coumaric acid, caffeic acid (Sigma-Aldrich, Merck Group, Darmstad), syringic acid (Alfa Aesar, Haverhill, MA, USA), chlorogenic acid (Cayman Chemical, Ann Arbor, MI, USA).

Ultrasound-assisted extraction of phenolic compounds

The phenolic compounds were extracted from honeysuckle by ultrasound-assisted extraction using an ultrasonic bath (Elmasonic P, Germany) at power $P = 580$ W and frequency 37 Hz of ultrasound. The temperature was maintained between 20-30°C by adding ice to the bath. The samples were placed in a volumetric flask with different volumes of solvent. After the extraction, the extract of polyphenols was separated from biomass by centrifugation, at 8500 rpm (6080 x g), 15 min, at 10°C, and used for the determination of the total polyphenols content (TPC), by the Folin Ciocalteu method, and for the assay of the antioxidant activity by DPPH, ABTS and FRAP methods (Xie et al., 2017).

Experimental Design

Response surface methodology is an approach to evaluate the relationship between responses

and independent variables, determining the optimal parameters and giving information about the predictive responses (Humbird et al., 2016).

According to other studies (Sun et al., 2011; Gam et al., 2020; Peng et al., 2020) the Box Behnken experimental design (BBD) with three-level and three factors were applied to determine the best combinations of extraction parameters for the extraction of the total polyphenol content (TPC) and the antioxidant activity (AOA) of extracts from flowers of honeysuckle.

The three factors selected for this experimental design were: the concentration of ethanol ($X_1 = 50, 73, 96\%$), the extraction time ($X_2 = 30, 45, 60$ min), and the ratio of liquid to solid (X_3 (L/S) = 10, 20, 30). The levels for each factor were coded with +1 which represents high, 0→middle and -1 →low (Table 1).

Table 1. The three-level quadratic model of three-factorial of Box-Behnken design

Test number	Ethanol concentration (%)	Time (min)	Liquid/solid ratio
1	50 (-1)	30 (-1)	20 (0)
2	96 (+1)	30 (-1)	20 (0)
3	50 (0)	60 (+1)	20 (0)
4	96 (+1)	60 (+1)	20 (0)
5	50 (-1)	45 (0)	10 (-1)
6	96 (+1)	45 (0)	10 (-1)
7	50 (-1)	45 (0)	30 (+1)
8	96 (+1)	45 (0)	30 (+1)
9	73 (0)	30 (-1)	10 (-1)
10	73 (0)	60 (+1)	10 (-1)
11	73 (0)	30 (-1)	30 (+1)
12	73 (0)	60 (+1)	30 (+1)
13	73 (0)	45 (0)	20 (0)
14	73 (0)	45 (0)	20 (0)
15	73 (0)	45 (0)	20 (0)
16	73 (0)	45 (0)	20 (0)
17	73 (0)	45(0)	20 (0)

The experimental design consisted of 17 experiments, 5 of which at the centre point were used to evaluate the experimental error. The general quadratic response model is described by equation 1.

The validity of the experimental model was evaluated by the determination of the regression coefficient of the model, by determination of adjusted R^2 and predicted R^2 , the significance (p) and lack of adjustment tests.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (1)$$

In this equation X_i, X_j represent the independent variables, $\beta_0, \beta_i, \beta_{ii}, \beta_{ij}$ are the regression coefficients for the intercept, linear, quadratic,

and interaction terms, respectively, k is the number of variables, and Y is the predicted response.

To evaluate the obtained model, the analysis of variance (ANOVA) was performed with Design Expert® software version 11.

The total polyphenol content

The total polyphenol content (TPC) of the extracts was determined by the Folin-Ciocalteu method. TPC was spectrophotometrically measured using a plate reader (CLARIOstar, BMG LABTECH, Germany) by the method described by (Kim et al., 2003). Briefly, 0.01 mL of standard gallic acid or sample solution was mixed with 0.09 mL H₂O d.d. and 0.01 mL of Folin Ciocalteu reagent, and after 5 minutes of mixing, 0.1 mL of 7% Na₂CO₃ and 0.04 mL H₂O d.d were added to the mixture. The absorbance was read at 765 nm after 60 minutes of incubation at room temperature. The calibration curve was in the range of 5-25 µg/mL gallic acid. The results were expressed as gallic acid equivalent/g mass of the sample.

The antioxidant activity

Radical scavenging activity by ABTS assay

The antioxidant method of neutralizing the ABTS radical was determined by the ABTS radical cation discolouration test (Proestos et al., 2013). ABTS⁺ was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, incubated in the dark at room temperature for 12-16 hours before use. The ABTS⁺ solution was then diluted with 96% ethanol to have an absorbance of 0.700 ± 0.04 at 734 nm. After adding 20 µl of sample to 180 µl of diluted ABTS⁺ solution, the absorbance was measured at 734 nm after 30 min. All measurements were performed three times. The results were expressed as mM Trolox / g dry weight (DW). Trolox was used as the standard substance, the calibration curve was made from the concentration range 0-150 µM Trolox.

Radical scavenging activity by DPPH assay

The method involves neutralizing the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) and was performed according to (Dudonné et al., 2009) with some modification. When DPPH reacts with an antioxidant compound,

which can donate hydrogen, it is reduced, varying in colour from purple to light yellow. Briefly, 100 µl of sample/standard was mixed with 100 µl of 0.3 mM DPPH solution in 99.6% (v/v) ethanol. The absorbance was read at $\lambda = 517$ nm after 30 min of reaction using a UV-Vis plate reader (CLARIOstar, BMG LABTECH, Germany). All measurements were performed three times. Trolox was used as the standard substance and the calibration curve was made from the concentration range 0.0125 mM - 0.15 mM, starting from a stock solution of Trolox 1 mM. The results were expressed as mM Trolox/g dry weight (DW).

Ferric-Ion Reducing Antioxidant Power (FRAP) Assay

The FRAP method is based on the ability of antioxidants to reduce the tripyridyltriazine - Fe³⁺ (Fe (III) -TPTZ) complex to the blue-coloured tripyridyltriazine-Fe²⁺ (Fe (II) -TPTZ) complex by the action of electron release by the antioxidant.

The determination of the antioxidant power of iron reduction was performed by the method described by (Benzie & Strain, 1999) with some modifications. The FRAP reagent was prepared by mixing 10 parts of 0.3 M acetate buffer pH 3.6 with one part of 10 mM TPTZ (in a solution of 40 mM HCl) and one part 20 mM FeCl₃ solution (10: 1: 1). An aliquot of 15 µL sample/standard solution was added to 285 µL FRAP reagent. The absorbance was read at 593 nm, after incubation for 30 minutes at 37°C in the dark. Trolox was used as the standard substance, the calibration curve was made from the concentration range 0-450 µM Trolox/mL. The results were expressed as mM Trolox/g dry weight (DW).

HPLC analysis

The phenolic acids from the optimised ethanolic extract of *Lonicera caprifolium*, obtained from optimisation of UAE, were identified using high pressure liquid chromatographic (HPLC) analysis. The phenolic acids were identified by comparison with the retention time of standards.

The HPLC analysis was performed on a Dionex Ultimate 3000 equipment and chromatograms were processed by Chromelleon 7.0 software. The chromatographic separation was performed

on a Luna Omega 5 μm Polar C18 100Å column (250 mm $\times$ 4.6 mm). The analysis was performed according to a method described by (Robbins & Bean, 2004). Briefly, the method involves using a gradient program with two solvents system (A - aqueous solution with 0.1% formic acid and B - methanol) which was applied as follows: 0-25 min. 5% B, 25-33 min. 30% B, 34-40 min. 5% B. The flow rate was set at 1.25 mL $\times$ min⁻¹ and the injection volume of 10 μL was used for the detection of phenolic acids at 280 nm.

The calibration curve consisted of several standard concentrations between 18.125-1000 $\mu\text{g/mL}$. The correlation coefficients (r^2) were above 0.9996, which indicated a good linearity.

Statistical analysis

As was already mentioned, the BBD models were used to evaluate the effect of each independent factors (concentration of ethanol, extraction time, and liquid to solid ratio) to the responses.

The calculations were performed using Design-Expert software 11 (State-Ease Inc., Minneapolis, MN, USA).

Analysis of variances (ANOVA) was applied to determine the statistical significance of the model and variables by determination of p -value, F -value, regression coefficient square terms, and to validate the model.

RESULTS AND DISCUSSIONS

The results of the UAE optimisation are summarized in Table 2, with values of TPC ranging between 392.093 and 1741.05 $\mu\text{g GAE/g DW}$, the values of antioxidant activity by DPPH method between 7.59 and 22.87 mM Trolox/g DW, by ABTS method between 29.41 and 162.37 mM Trolox/g DW, and by FRAP method between 39.78 and 204.06 mM Trolox/g DW.

The results of the statistical analysis are presented in Table 3. In the case of TPC, the F -value (101.13) and the p -value ($p < 0.0001$) indicate the model was significant.

The data shown in Table 3 indicate that the ethanol concentration was a highly significant factor ($p < 0.0001$) for TPC.

Also, the reduced quadratic model was significant for the response TPC, with a good regression coefficient $R^2 = 0.9353$ (more than

93% of variation can be predicted by the model).

Table 2. Experimental results

Test number	TPC $\mu\text{g GAE/g DW}$	DPPH mM Trolox/g DW	ABTS mM Trolox/g DW	FRAP mM Trolox/g DW
1	1387.41 $\pm$ 54.10	13.32 $\pm$ 0.62	61.99 $\pm$ 3.40	185.06 $\pm$ 9.45
2	1365.2 $\pm$ 21.32	21.34 $\pm$ 1.23	78.65 $\pm$ 0.62	204.06 $\pm$ 0.67
3	471.69 $\pm$ 24.82	19.09 $\pm$ 0.87	39.81 $\pm$ 0.59	48.98 $\pm$ 9.68
4	652.56 $\pm$ 27.10	17.06 $\pm$ 1.20	44.12 $\pm$ 3.26	71.53 $\pm$ 0.58
5	1330.59 $\pm$ 53.95	16.20 $\pm$ 0.48	154.6 $\pm$ 2.73	171.33 $\pm$ 4.85
6	1551.28 $\pm$ 31.32	22.10 $\pm$ 0.73	57.38 $\pm$ 0.50	176.12 $\pm$ 1.32
7	392.093 $\pm$ 69.41	7.911 $\pm$ 2.82	29.41 $\pm$ 4.39	39.78 $\pm$ 12.35
8	1507.15 $\pm$ 29.01	8.90 $\pm$ 0.32	110.52 $\pm$ 1.6	145.96 $\pm$ 4.075
9	1606.68 $\pm$ 97.88	22.87 $\pm$ 1.72	93.31 $\pm$ 4.03	163.47 $\pm$ 6.35
10	1486.84 $\pm$ 66.31	17.59 $\pm$ 1.34	153.49 $\pm$ 1.54	168.78 $\pm$ 5.84
11	1741.05 $\pm$ 39.59	7.59 $\pm$ 0.46	98.3 $\pm$ 0.63	156.65 $\pm$ 10.67
12	1264.3 $\pm$ 72.76	15.85 $\pm$ 1.19	149.93 $\pm$ 0.61	159.37 $\pm$ 13.30
13	1530.4 $\pm$ 86.83	17.35 $\pm$ 1.21	162.37 $\pm$ 5.99	175.25 $\pm$ 10.36
14	1549.34 $\pm$ 64.94	17.75 $\pm$ 0.77	61.75 $\pm$ 2.11	172.31 $\pm$ 4.17
15	1475.47 $\pm$ 77.78	16.74 $\pm$ 1.07	161.49 $\pm$ 2.03	178 $\pm$ 3.27
16	1571.16 $\pm$ 53.07	16.59 $\pm$ 0.81	53.047 $\pm$ 4.78	72.29 $\pm$ 14.52
17	1399.67 $\pm$ 28.02	18.69 $\pm$ 0.51	108.41 $\pm$ 2.03	136.84 $\pm$ 8.73

The difference between the predicted R^2 of 0.9008 and the adjusted R^2 of 0.9260 was less than 0.2. The lack of fit F -value of 1.11 implies the lack of fit is not significant relative to the pure error.

In the case of AOA by ABTS and FRAP methods, the reduced quadratic model was significant with F -value 93.15 and 147.61, respectively, and regression coefficient $R^2 = 0.9864$ (ABTS) and $R^2 = 0.9888$ (FRAP). For both methods, the predicted R^2 was in reasonable agreement with the adjusted R^2 .

Also, the data presented in Table 3 show that the ethanol concentration and liquid to solid ratio were significant for the antioxidant activity of the extracts from flowers of honeysuckle by all methods studied. In the case of AOA by the DPPH method, the linear model was fit better than others, with regression coefficient $R^2 = 0.9538$, model F -value 89.48 and model p -value < 0.0001 .

Table 3. Analysis of variance (ANOVA) for the selected factorial model

Source	TPC				AOA FRAP			
	Sum of squares	Mean square	F-value	p-value	Sum of squares	Mean square	F-value	p-value
Model	2.826E+06	1.413E+06	101.13	< 0.0001	41239.52	6873.25	147.61	< 0.0001
X ₁	2.144E+06	2.144E+06	153.48	< 0.0001	26168.89	26168.89	562.01	< 0.0001
X ₂	-	-	-	-	903.13	903.13	19.40	0.0013
X ₃	-	-	-	-	2336.06	2336.06	50.17	< 0.0001
Interaction								
X ₂ -X ₃	-	-	-	-	247.60	247.60	5.32	0.0438
Quadratic								
X ₁ ²	6.815E+05	6.815E+05	48.78	< 0.0001	11075.98	11075.98	237.87	< 0.0001
X ₃ ²	-	-	-	-	277.36	277.36	5.96	0.0348
Residuals	1.956E+05	13969.37	-	-	465.63	46.56	-	-
Lack of Fit	1.436E+05	14363.24	1.11	0.5026	259.33	43.22	0.8380	0.5976
Pure Error	51938.78	12984.69	-	-	206.31	51.58	-	-
Source	AOA DPPH				AOA ABTS			
	Sum of squares	Mean square	F-value	p-value	Sum of squares	Mean square	F-value	p-value
Model	466.62	155.54	89.48	< 0.0001	34483.99	4926.28	93.15	< 0.0001
X ₁	12.21	12.21	7.02	0.0200	1597.00	1597.00	30.20	0.0004
X ₂	7.53	7.53	4.33	0.0577	8.02	8.02	0.1516	0.7061
X ₃	446.88	446.88	257.09	< 0.0001	516.13	516.13	9.76	0.0122
Interaction								
X ₁ - X ₃	-	-	-	-	1041.85	1041.85	19.70	0.0016
Quadratic								
X ₁ ²	-	-	-	-	21420.02	21420.02	405.02	< 0.0001
X ₂ ²	-	-	-	-	4623.17	4623.17	87.42	< 0.0001
X ₃ ²	-	-	-	-	2741.83	2741.83	51.84	< 0.0001
Residuals	22.60	1.74	-	-	475.98	52.89	Tan	-
Lack of Fit	20.42	2.27	4.16	0.0916	360.82	72.16	2.51	0.1970
Pure Error	2.18	0.5454	-	-	115.16	28.79	-	-

X₁ - ethanol concentration, X₂- extraction time, X₃- liquid to solid ratio.

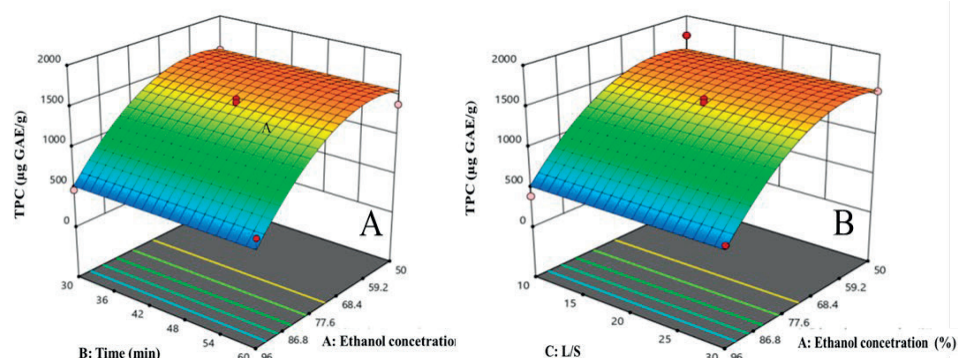


Figure 1. Response surface showing the effect of ethanol concentration, time, and liquid to solid ratio on the total polyphenol content

Figure 1 represents graphically the interaction between variables and the effects on the TPC value. As reported in Table 3, the effect of the ethanol concentration on TPC is significant ($p < 0.0001$) and the TPC value decreases with the increase of ethanol concentration beyond 50%. Similar behaviour was recently obtained for the polyphenols extraction from *Lonicera japonicum* (Fan et al., 2019) and was reported also in the case of extractions from other plants (Sun et al., 2011; Arteaga-Crespo et al., 2020; Gam et al., 2020).

In general, maximum TPC is obtained at 50-70% ethanol, sometimes higher. At too low ethanol concentrations the polarity of the solvent is too high and the viscosity is increased, while at too high ethanol concentrations the polarity of the solvent decreases too much for efficient extraction of polyphenols.

The results presented in Table 3 and Figure 1 indicate that time and solid to liquid ratio were insignificant to the TPC value and that there was no interaction between them.

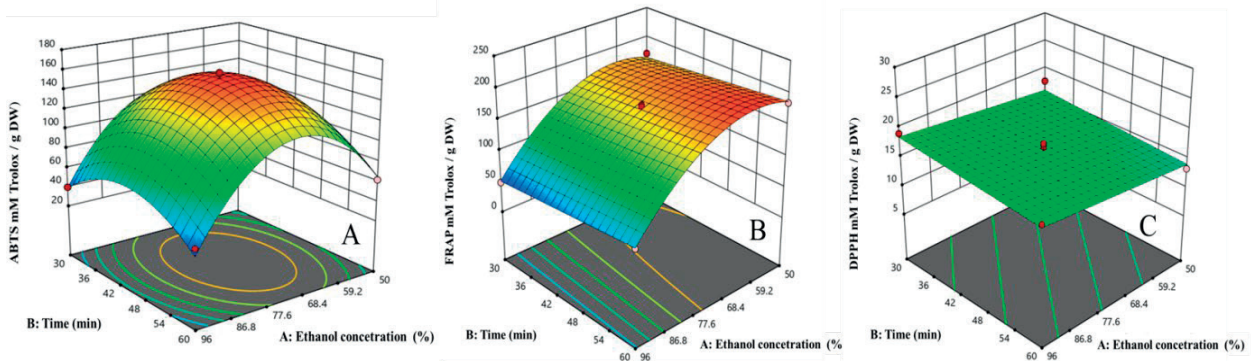


Figure 2. Response surface plots for the effect of ethanol concentration and extraction time on the antioxidant activity by ABTS method (A), by FRAP method (B), by DPPH method (C)

Figure 2 shows the AOA by ABTS, FRAP and DPPH, as a function of ethanol concentration and extraction time. The ethanol concentration influences the AOA by all methods, but in the case of DPPH, the ethanol concentration presented a lower level of significance compared to the other two methods (Figure 2C) as reported also by ANOVA p-value of 0.02 (Table 3).

The surface response plot demonstrated a significant quadratic effect of ethanol concentration on AOA measured by ABTS and FRAP method. Figure 2A shows that the

maximum AOA by ABTS method (162.37 mM Trolox/g DW) was obtained at 73% ethanol concentration and 45 min extraction time, after which the tendency became downwards in both directions. Also, figure 2B shows that the maximum value of AOA by FRAP assay was obtained at 73% ethanol concentration and 45 min extraction time, which it represents the inflexion point of the 3D curve. This trend data can be also observed in the results of other authors (Gam et al., 2020).

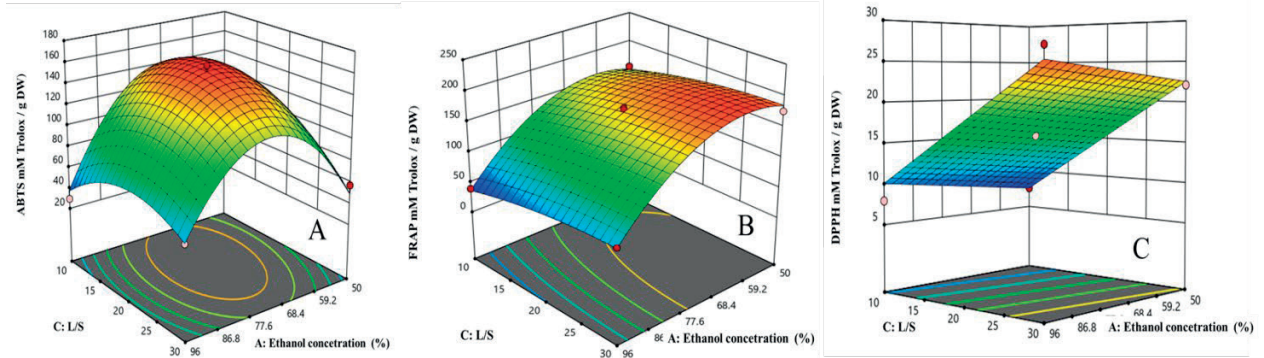


Figure 3. Response surface plots for the effect of ethanol concentration and liquid to solid ratio on the antioxidant activity by ABTS method (A), by FRAP method (B), by DPPH method (C)

Similar behaviour was observed in the case of the 3D surface representation as a function of L/S and ethanol concentration (Figure 3). In the case of DPPH, the L/S had a more significant effect than the extraction time. The DPPH antioxidant activity increased linearly with increasing L/S (Figure 3C).

The second-order polynomial equation in terms of code factors determined from the experimental results is presented as:

$$Y_{TPC} = 1440.70 - 517.7X_1 - 401.13X_1^2 \quad (2)$$

$$Y_{ABTS} = 156.38 - 14.13X_1 - 1.00X_2 - 8X_3 + 16.14X_1X_3 - 71.32X_1^2 - 33.14X_2^2 - 25.52X_3^2 \quad (3)$$

$$Y_{DPPH} = 16.29 + 1.24X_1 - 0.9704X_2 + 7.47X_3 \quad (4)$$

$$Y_{FRAP} = 170.61 - 57.19X_1 + 10.63X_2 + 17.09X_3 + 7.87X_2X_3 - 51.22X_1^2 - 8.11X_3^2 \quad (5)$$

We calculated the Pearson's correlation coefficients (r) between response variables (Table 4). The behaviour of FRAP was similar to that of TPC, both methods being highly positively correlated ($r = 0.768$). Significant correlation was found between ABTS and

FRAP ($r = 0.589$) and between TPC and ABTS ($r = 0.493$). These results suggest that most of the extracted polyphenols detected by TPC were involved in these AOA. The lowest correlation was found between DPPH and ABTS assay ($r = 0.034$).

Table 4. Pearson correlation coefficients

	TPC $\mu\text{g GAE/g DW}$	DPPH mM Trolox/g DW	ABTS mM Trolox / g DW	FRAP mM Trolox / g DW
TPC $\mu\text{g GAE/g DW}$	1	0.144	0.493	0.768
DPPH mM Trolox/g DW	0.144	1	0.034	0.268
ABTS mM Trolox/g DW	0.493	0.034	1	0.589
FRAP mM Trolox/g DW	0.768	0.268	0.589	1

Differences in correlations were reported also by others and seem to depend on the substrate used for extraction (Andres et al., 2013). Andres et al. obtained correlation between TPC and ABTS and between DPPH and reducing power (RP), but not between these two groups, for hydroalcoholic extraction of brewers' spent grain (BSG). They explained these results in terms of the different solubilities of the AOA substrates. DPPH can be solubilised only in organic solvents, therefore mostly the lipophilic antioxidants contribute to the reaction. ABTS is soluble in both aqueous and organic solvents and it is usually measured in hydroalcoholic solution, therefore both hydrophilic and lipophilic compounds contribute to AOA.

In our case, TPC and FRAP are the most similar in terms of the solvent used (aqueous solution) which could be one explanation for the positive correlation between the two methods. The response of ABTS is more complex, probably because of the significant contribution of both hydrophilic and lipophilic fractions. In the case of DPPH, only the more lipophilic fraction has a significant contribution, which correlates with the slight

increase in the DPPH activity when the ethanol concentration increases. The significant and linear influence of L/S suggests that the extraction of the more lipophilic fraction is not saturated in the L/S interval selected.

Another possible explanation related to the differences between the antioxidant activities determined by the different methods is related to the mechanisms characteristic of each antioxidant assay method. There are several mechanisms involved in antioxidant activity, such as hydrogen atom transfer (HAT), sequential proton loss–electron transfer (SPLET), single-electron transfer–proton transfer (SET-PT) mechanisms (Galano et al., 2016). Most of the antioxidant assays which we used are related to electron transfer, SPLET or SET-PT (Apak et al., 2016). However, recently it was suggested that DPPH assay could have HAT, SET-PT and SPLET mechanisms in different micro-environments (Chen et al., 2020). Therefore, the lack of correlation of DPPH assay with other types of assays could be explained by the predominance of HAT mechanism (Tošović et al., 2017) for DPPH in our assays.

A third explanation for the different responses could be the differences in the extraction composition as a function of variables. This aspect needs a thoroughly investigation of the composition response in terms of different classes of compounds and it is planned to be further investigated.

Selection of the optimum factors and estimation of the optimum responses

The desirability function method was employed to optimise the extraction conditions to obtain maximum extraction yield of TPC and AOA by all methods as described in Table 5.

Optimal conditions were established to be 61.079% ethanol, 32.283 min extraction time at a liquid/solid ratio of 18.906.

Table 5. Optimal factors and predictive responses

Ethanol concentration, %	Time, min	L/S	TPC $\mu\text{g GAE/g DW}$	DPPH mM Trolox/ g DW	ABTS mM Trolox / g DW	FRAP mM Trolox / g DW	Desirability
61.079	32.283	18.906	1601.266	15.656	123.060	176.252	0.705

HPLC analysis

The phenolic acids from the optimized ultrasound extract of *L. caprifolium* flowers were identified by HPLC, by comparing with the retention time (RT) of standards.

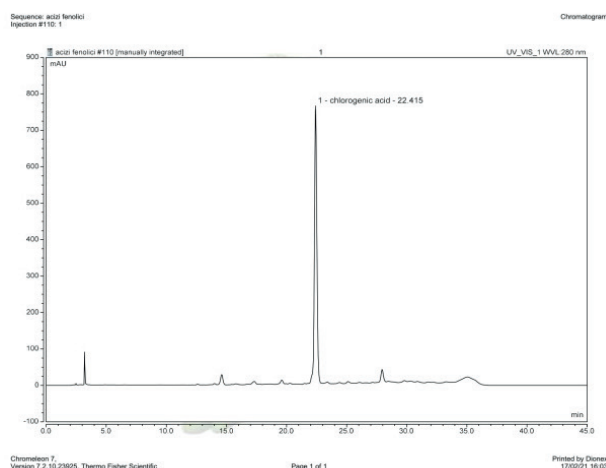


Figure 4. HPLC chromatogram of honeysuckle flower extract obtained by UAE extraction at optimised conditions

The HPLC chromatogram showed one major peak at RT 22.415 min, which was identified and quantified as chlorogenic acid -1331.91 µg/g DW (Figure 4). Chlorogenic acid was previously shown to be the main phenolic acid in other *Lonicera* species (Wang et al., 2009; Yuan et al., 2014; Chaowuttikul et al., 2020). Antioxidant mechanism of chlorogenic acid in conditions similar to our AOA assay is predominantly of electron transfer - SPLET (Tošović et al., 2017). Such mechanism could be an explanation for different correlation coefficients between various AOA assays of extracts of *L. caprifolium* flowers (Table 4).

CONCLUSIONS

The UAE of polyphenols from flowers of *Lonicera caprifolium* was optimised using DoE and RSM in Design Expert software version 11. A BBD three-level, three-factor (ethanol concentration, extraction time, liquid to solid ratio) was applied to obtain the optimal condition with regards to highest yield of TPC and AOA. AOA was assessed through three colourimetric methods namely DPPH, ABTS and FRAP. The highly significant factor for polyphenol content and AOA was the ethanol concentration, while the other two were less significant for the extraction of the maximum

total polyphenolic compounds and the maximum AOA of the extracts. The correlations between TPC and AOA depended on the method of AOA, one possible explanation being the reagent solubility. The optimal extract was analysed by HPLC to identify the phenolic acids, and the main phenolic acid was chlorogenic acid, similar to other *Lonicera* species.

All of these facts point to the possible use of this extract in different products such as cosmetics and nutraceuticals.

ACKNOWLEDGEMENTS

This work was supported by project POC-A1-A1.2.3-G-2015 - P_40-352 - SECVENT 81/2016, “*Sequential processes of closing the side streams from bioeconomy and innovative (bio)products resulting from it*”, funded by cohesion funds of the European Union. Subsidiary project 5544/2020 Meli-STIM. We thank Dr. Bogdan Trică for the help in using Design Expert software.

REFERENCES

- Abbas, M., Saeed, F., Anjum, F.M., Afzaal, M., Tufail, T., Bashir, M.S., . . . Suleria, H.A.R. (2017). Natural polyphenols: An overview. *International Journal of Food Properties*, 20(8), 1689-1699.
- Ali, A., Lim, X.Y., Chong, C.H., Mah, S.H. & Chua, B.L. (2018). Optimization of ultrasound-assisted extraction of natural antioxidants from Piper betle using response surface methodology. *LWT*, 89, 681-688.
- Andres, A.I., Petron, M., Lopez, A.M. & Timon, M.L. (2020). Optimization of Extraction Conditions to Improve Phenolic Content and In Vitro Antioxidant Activity in Craft Brewers' Spent Grain Using Response Surface Methodology (RSM). *Foods*, 9(10), 1398.
- Apak, R., Özyürek, M., Güçlü, K. & Çapanoğlu, E. (2016). Antioxidant Activity/Capacity Measurement. 1. Classification, Physicochemical Principles, Mechanisms, and Electron Transfer (ET)-Based Assays. *Journal of Agricultural and Food Chemistry*, 64(5), 997-1027.
- Arteaga-Crespo, Y., Radice, M., Bravo-Sanchez, L.R., García-Quintana, Y. & Scalvenzi, L. (2020). Optimisation of ultrasound-assisted extraction of phenolic antioxidants from Ilex guayusa Loes. leaves using response surface methodology. *Heliyon*, 6(1), e03043.
- Benzie, I.F.F. & Strain, J.J. (1999). [2] Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and

- modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In *Methods in Enzymology*, Vol. 299, 15-27, Academic Press.
- Chaowuttikul, C., Palanuvej, C. & Ruangrunsi, N. (2020). Quantification of chlorogenic acid, rosmarinic acid, and caffeic acid contents in selected Thai medicinal plants using RP-HPLC-DAD. *Brazilian Journal of Pharmaceutical Sciences*, 56.
- Chemat, F., Rombaut, N., Sicaire, A.-G., Meullemiestre, A., Fabiano-Tixier, A.-S. & Abert-Vian, M. (2017). Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics Sonochemistry*, 34, 540-560.
- Chen, J., Yang, J., Ma, L., Li, J., Shahzad, N. & Kim, C.K. (2020). Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Scientific Reports*, 10(1), 2611.
- Dudonné, S., Vitrac, X., Coutière, P., Woillez, M. & Mérillon, J.-M. (2009). Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH, ABTS, FRAP, SOD, and ORAC Assays. *Journal of Agricultural and Food Chemistry*, 57(5), 1768-1774.
- Fan, Z., Li, L., Bai, X., Zhang, H., Liu, Q., Zhang, H., . . . Moyo, R. (2019). Extraction optimization, antioxidant activity, and tyrosinase inhibitory capacity of polyphenols from *Lonicera japonica*. *Food Science & Nutrition*, 7(5), 1786-1794.
- Galano, A., Mazzone, G., Alvarez-Diduk, R., Marino, T., Alvarez-Idaboy, J.R. & Russo, N. (2016). Food antioxidants: chemical insights at the molecular level. *Annual review of food science and technology*, 7, 335-352.
- Gam, D.H., Kim, S.Y. & Kim, J.W. (2020). Optimization of Ultrasound-Assisted Extraction Condition for Phenolic Compounds, Antioxidant Activity, and Epigallocatechin Gallate in Lipid-Extracted Microalgae. *Molecules*, 25(3).
- Humbird, D. & Fei, Q. (2016). Chapter 20 - Scale-Up Considerations for Biofuels. In C. A. Eckert & C. T. Trinh (Eds.), *Biotechnology for Biofuel Production and Optimization*, 513-537, Amsterdam: Elsevier.
- Ilies, D.C., Radulescu, V. & Duțu, L.E. (2013). Volatile constituents from the flowers of two species of honeysuckle (*Lonicera japonica* and *Lonicera caprifolium*). *Farmacia*, 62, 194-201.
- Kim, D.-O., Jeong, S.W. & Lee, C.Y. (2003). Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. *Food Chemistry*, 81(3), 321-326.
- Nichita, C., Neagu, G., Cucu, A., Vulturescu, V., Vifor, Ș. & Berteșteanu, G. (2016). Antioxidative properties of *Plantago lanceolata* L. extracts evaluated by chemiluminescence method. *AgroLife Scientific Journal* 5(2).
- Peng, X., Yang, G., Shi, Y., Zhou, Y., Zhang, M. & Li, S. (2020). Box-Behnken design based statistical modeling for the extraction and physicochemical properties of pectin from sunflower heads and the comparison with commercial low-methoxyl pectin. *Scientific Reports*, 10(1).
- Proestos, C., Lytoudi, K., Mavromelanidou, O.K., Zoumpoulakis, P. & Sinanoglou, V.J. (2013). Antioxidant capacity of selected plant extracts and their essential oils. *Antioxidants*, 2(1), 11-22.
- Proteggente, A.R., Pannala, A.S., Paganga, G., Buren, L.V., Wagner, E., Wiseman, S., . . . Rice-Evans, C.A. (2002). The Antioxidant Activity of Regularly Consumed Fruit and Vegetables Reflects their Phenolic and Vitamin C Composition. *Free Radical Research*, 36(2), 217-233.
- Robbins, R.J. & Bean, S.R. (2004). Development of a quantitative high-performance liquid chromatography-photodiode array detection measurement system for phenolic acids. *Journal of Chromatography A*, 1038(1), 97-105.
- Rodriguez-Casado, A. (2016). The Health Potential of Fruits and Vegetables Phytochemicals: Notable Examples. *Critical Reviews in Food Science and Nutrition*, 56(7), 1097-1107.
- Seo, O.N., Kim, G.-S., Park, S., Lee, J.H., Kim, Y.-H., Lee, W.S., . . . Shin, S.C. (2012). Determination of polyphenol components of *Lonicera japonica* Thunb. using liquid chromatography-tandem mass spectrometry: Contribution to the overall antioxidant activity. *Food Chemistry*, 134(1), 572-577.
- Sun, Y., Xu, W., Zhang, W., Hu, Q. & Zeng, X. (2011). Optimizing the extraction of phenolic antioxidants from kudingcha made from *Ilex kudingcha* C.J. Tseng by using response surface methodology. *Separation and Purification Technology*, 78(3), 311-320.
- Tošović, J., Marković, S., Dimitrić Marković, J.M., Mojović, M. & Milenković, D. (2017). Antioxidative mechanisms in chlorogenic acid. *Food Chemistry*, 237, 390-398.
- Vamanu, E. & Nita, S. (2013). Antioxidant Capacity and the Correlation with Major Phenolic Compounds, Anthocyanin, and Tocopherol Content in Various Extracts from the Wild Edible *Boletus edulis* Mushroom. *BioMed Research International*, 2013, 313905.
- Wang, L.-m., Li, M.-t., Yan, Y.-y., Ao, M.-z., Wu, G. & Yu, L.-j. (2009). Influence of flowering stage of *Lonicera japonica* Thunb. on variation in volatiles and chlorogenic acid. *Journal of the Science of Food and Agriculture*, 89(6), 953-957.
- Xie, Y., Huang, Q., Wang, Z., Cao, H. & Zhang, D. (2017). Structure-activity relationships of cinnamaldehyde and eugenol derivatives against plant pathogenic fungi. *Industrial Crops and Products*, 97, 388-394.
- Yuan, Y., Wang, Z., Jiang, C., Wang, X. & Huang, L. (2014). Exploiting genes and functional diversity of chlorogenic acid and luteolin biosyntheses in *Lonicera japonica* and their substitutes. *Gene*, 534(2), 408-416.