Optimization of Ultrasound Assisted Extraction of Antioxidant Compounds from Marjoram (Origanum majorana L.) Using Response Surface Methodology

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Optimization of ultrasound assisted extraction of antioxidant compounds from Marjoram (\textit{Origanum majorana} L.) using response surface methodology

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ABSTRACT

The present study optimized the ultrasound assisted extraction (UAE) conditions to maximize the antioxidant activity [Ferric ion Reducing Antioxidant Power (FRAP)], total phenol content (TP) and content of individual polyphenols of the extracts from four Lamiaceae herbs namely marjoram, oregano, rosemary and sage. Optimal conditions with regard to amplitude of sonication (24.4–61.0 μm) and extraction temperature (15-35 °C) and time (5-15 min) were identified using response surface methodology (RSM). The results showed that the combined treatment of 61 μm, 35 °C and 15 min was optimal for maximizing TP, FRAP, rosmarinic acid, luteolin-7-O-glucoside, apigenin-7-O-glucoside, caffeic acid, carnosic acid and carnosol values of the extracts. The predicted values from the quadratic polynomial equation were in close agreement with the actual experimental values with low average mean deviation (E%) ranging from 0.45 to 1.55 %. The extraction yields of the optimal UAE were significantly (p<0.05) higher than solid/liquid extracts. Predicted models were highly significant (p<0.05) for all the parameters studied with high regression coefficients (R²) ranging from 0.710 to 0.989.

KEYWORDS: Antioxidant, spice, ultrasound assisted extraction, total phenols, RSM
INTRODUCTION

Marjoram has been traditionally used for the treatment of gastrointestinal disturbances, cough and bronchial diseases. Marjoram is also applied topically to relieve symptoms of the common cold, such as nasal congestion and in mouthwashes for oral hygiene (1). Several studies reported that methanolic extracts of marjoram had high antioxidant capacity (2, 3) mostly due to the polyphenolic compounds present in them. Recently, interest has increased considerably in naturally occurring antioxidants for use in foods or medicinal materials as replacements for synthetic antioxidants such as BHA and BHT, whose use is being restricted due to concerns over safety (4, 5). Natural antioxidants can protect the human body from free radicals and could retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods (6-8). A host of potentially beneficial physiological effects have been postulated for antioxidants over the past three decades which are supported by extensive animal studies. Among these are beneficial influences on lipid metabolism, efficacy as anti-diabetic, ability to stimulate digestion, antioxidant property, anti-carcinogenic and anti-inflammatory potential (9, 10).

Oxidation of polyunsaturated fatty acids not only lowers the nutritional value of food (11), but is also associated with cell membrane damage, aging, heart disease and cancer in living organisms (12). Therefore the addition of natural antioxidants to food products has become popular as a means of increasing shelf life and to reduce wastage and nutritional losses by inhibiting and delaying oxidation (13). However, an efficient extraction technique is required in order to harvest the benefits of natural antioxidants present in marjoram. A number of techniques are available for the extraction of natural antioxidants from plants, including ultrasound-assisted extraction, supercritical fluid
extraction, microwave-assisted extraction, and solvent extraction (14, 15). Among these, ultrasound-assisted extraction (UAE) offers an inexpensive, environmentally friendly, less time consuming and efficient alternative to conventional extraction techniques. The enhancement in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitations produced in the solvent by the passage of an ultrasound wave (16, 17). Ultrasound also offers a mechanical effect allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between the solid and liquid phase, and as a result, the solute quickly diffuses from the solid phase to the solvent (18, 19).

In this study, UAE parameters such as extraction temperature, extraction time and amplitude of ultrasound were optimized using response surface methodology (RSM), by employing a Box-Behnken design to maximize extraction of antioxidant polyphenolic compounds from marjoram.

MATERIALS AND METHODS

Samples and reagents. Dried and ground marjoram leaf was provided by AllinAll Ingredients Limited, Dublin 12. The country of origin of the spices was Turkey. The plants were grown in sunny and well drained land with annual rainfall of around 15 inches. As per the product specifications the samples were air dried at ambient temperature (~ 23 °C) after heat treatment (steam sterilization at 120 °C for 30 sec). Folin-Ciocalteu Reagent, gallic acid, sodium acetate anhydrous, ferric chloride hexahydrate, 2,4,6-Tri(2-pyridyl)-s-triazine, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-
carboxylic acid, sodium carbonate, caffeic acid, rosmarinic acid, luteolin-7-O-glucoside, apigenin-7-O-glucoside, carnosic acid and carnosol were purchased from Sigma-Aldrich.

**Sonication treatment.** A 1500W ultrasonic processor (VC 1500, Sonics and Materials Inc., Newtown, USA) with a 19 mm diameter probe was used for sonication. Samples were processed at a constant frequency of 20 kHz. The energy input was controlled by setting the amplitude of the sonicator probe. Extrinsic parameters of amplitude (24.4–61.0 μm), temperature (15–35 °C) and processing time (5–15 min) were varied with pulse durations of 5 s on and 5 s off. Dried leaf particles of marjoram (1 g) were placed in a 50 mL jacketed vessel through which water was circulated at 15±0.5, 25±0.5 and 35±0.5 °C with a flow rate of 0.5 L/min. Sonication at the desired amplitude level was started once the set temperature was reached. The ultrasound probe was submerged to a depth of 25 mm in the sample. All treatments were carried out in triplicate.

**Conventional solid/liquid extraction.** Solid/liquid extractions were carried out according to the method of Shan et al. (3) with slight modifications. Briefly, dried and ground samples (0.5 g) were homogenized for 1 min at 24,000 rpm using an Ultra-Turrax T-25 Tissue homogenizer (Janke & Kunkel, IKA®-Labortechnik, Saufen, Germany) in 25 mL of 80% methanol at room temperature (~23 °C). The homogenized sample suspension was shaken for 3 hours with a V400 Multitude Vortexer (Alpha laboratories, North York, Canada) at 1,500 rpm at room temperature (≥ 25°C). The sample suspension was then centrifuged for 15 min at 2,000 g (MSE Mistral 3000i, Sanyo Gallenkamp, Leicestershire, UK) and immediately filtered through 0.45 μm polytetrafluoroethylene
(PTFE) filters. The extracts were kept at -20 °C until subsequent analysis. The experiment was performed in two batches which included three replications of each sample.

**Determination of total phenol (TP).** The total phenolic content was determined using Folin-Ciocalteu Reagent (FCR) as described by Singelton et al. (20). The experiment was performed in two batches which included three replications of each sample and standard. Methanolic gallic acid solutions (10-400 mg/L) were used as standards. In each replicate, 100 µL of the appropriately diluted sample extract, 100 µL methanol, 100 µL FCR and finally 700 µL Na₂CO₃ (20%) were added together and vortexed. The mixture was incubated for 20 min in the dark and room temperature. After incubation the mixture was centrifuged at 13,000 rpm for 3 min. The absorbance of the supernatant was measured at 735 nm by spectrophotometer. The total phenolic content was expressed as gallic acid equivalent (GAE)/100 g dry weight (DW) of the sample.

**Ferric ion reducing antioxidant power (FRAP) assay.** The FRAP assay was carried out as described by Stratil et al. (21) with slight modifications. The FRAP reagent was prepared by mixing 38 mM sodium acetate anhydrous in distilled water pH 3.6, 20 mM FeCl₃.6H₂O in distilled water and 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl in a proportion of 10:1:1. This reagent was freshly prepared before each experiment. To each sample 100 µL of appropriately diluted sample extract and 900 µL of FRAP reagent was added and the mixture was incubated at 37 °C for 40 min in the dark. In the case of the blank 100 µL of methanol was added to 900 µL of FRAP reagent.
The absorbance of the resulting solution was measured at 593 nm by spectrophotometer. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (a synthetic antioxidant) at concentrations from 0.1 mM-0.4 mM was used as a reference antioxidant standard. FRAP values were expressed as g Trolox/100 g DW of the sample.

**HPLC analysis of the extracts.** Reversed phase high performance liquid chromatography (RP-HPLC) of the filtered sample extracts were carried out according to the method of Tsao and Yang (22). The chromatographic system (Shimadzu-Model no SPD-M10A VP, Mason Technology, Dublin 8, Ireland) consisted of a pump, a vacuum degasser, a Diode-Array Detector and was controlled through EZ Start 7.3 software (Shimadzu) at 37 °C. An Agilent C18 column (15 cm × 4.6 cm, 5 μm, Agilent Technologies., USA) was utilised with a binary mobile phase of 6 % acetic acid in 2 mM sodium acetate (final pH 2.55, v/v, solvent A) and acetonitrile (solvent B). Solvent A was prepared first by making 2 mM sodium acetate water solution, which was then mixed with acetic acid at a ratio of 94:6 by volume. All solvents were filtered through a 0.45 μm membrane filter prior to analysis. The flow rate was kept constant at 1.0 mL/min for a total run time of 80 min. The following gradient program was carried out: 0-15% B in 45 min, 15-30% B in 15 min, 30-50% B in 5 min, 50-100% B in 5 min and 100-0% B in 5 min. The injection volume for all the samples was 10 μL. All the standards for quantification purposes were dissolved in methanol. The detection wavelength of 280 nm was used for the detection of carnosol and carnosic acid. Rosmarinic acid, caffeic acid and apigenin-7-O-glucoside were detected at 320 nm while luteilon-7-O-glucoside was detected at 360 nm. Identification of the compounds was achieved by comparing their
retention times and UV-Vis spectra with those of authenticated standards by using the inline DAD with a 3D feature. Results are expressed as mean values of three assays for each replicated experiment.

**Experimental design and data analysis.** Polynomial regression equations were developed to describe the effects of the 3 independent processing parameters; ultrasound amplitude ($X_1$, μm), extraction temperature ($X_2$, °C) and processing time ($X_3$, min) on total phenol (TP), antioxidant activity as measured by FRAP and different polyphenolic compounds such as rosmarinic acid, caffeic acid, luteolin-7-O-glucoside, apigenin-7-O-glucoside, carnosol and carnosic acid. Independent variables of amplitude level ($X_1$) (24.4, 42.7, and 61 μm), temperature ($X_2$) (15, 25, 35 °C), and processing time ($X_3$) (5, 10 and 15 min) were varied to investigate the effects on dependent variables mentioned above. The general form of the quadratic polynomial model regression equation employed in this study is presented in Eq. 1. By using this equation, linear ($X_1$, $X_2$, $X_3$), quadratic ($X_1^2$, $X_2^2$, $X_3^2$) and interactive ($X_1X_2$, $X_1X_3$, $X_2X_3$) effects of independent variables, temperature ($X_1$), amplitude level ($X_2$), and time ($X_3$) on dependent variable ($Y$) were determined.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad \ldots \ldots \quad \text{(Eq. 1)}$$

Where $Y$ is the predicted response; $\beta_0$ the constant (intercept); $\beta_i$ the linear coefficient; $\beta_{ii}$ the quadratic coefficient and $\beta_{ij}$ is the cross product coefficient. $X_i$ and $X_j$ are independent variables. The response surface regression was used to analyze the experimental data using Design Expert Version 7.1.3 software (Stat-Ease, Inc., Minneapolis, MN). Two
dimensional contour plots were developed while holding a variable constant in the second order polynomial models. All processing trials were conducted in triplicate.

**Model validation.** The predictive performance of the developed models describing the combined effect amplitude ($X_1$), temperature ($X_2$) and time ($X_3$) on independent variables (FRAP, TP, rosmarinic acid, caffeic acid, luteolin-7-$O$-glucoside, apigenin-7-$O$-glucoside, carnosol and carnosic acid) of marjoram were validated with optimal extraction conditions as predicted by the design.

The criterion used to characterize the fitting efficiency of the data to the model was the multiple correlation coefficients ($R^2$) and their average mean deviation ($E$, Eq. 2).

\[ E(\%) = \frac{1}{n_e} \sum_{i=1}^{n_e} \left| \frac{V_E - V_P}{V_E} \right| \times 100 \]

\[ \text{……………..(Eq. 2)} \]

where, $n_e$ is the number of experimental data, $V_E$ is the experimental value and $V_P$ is the predicted value.

**RESULTS AND DISCUSSION**

**Effect of thermosonication on total phenol content**

Figure 1A presents the contour plot showing the effect of three different parameters of UAE such as ultrasound amplitude, temperature, and time on the total phenol content of marjoram, oregano, rosemary and sage extracts. All the three factors had significant ($p<0.05$) positive effect on the total phenol content of these extracts. Among the factors, amplitude showed the highest effect followed by temperature and time except in oregano where the order of effect of the parameters was amplitude $>$ time $>$ temperature. An increase in temperature increases target compound solubility, solvent diffusion rate and
mass transfer, while solvent viscosity and surface tension decrease (Hossain et al., 2010). Reduced viscosity and surface tension facilitates the solvent to access deeper into sample matrix which enhances extraction efficiency by exposing more surface area of the sample to solvents used. Higher amplitude of ultrasound could have damaged more cell walls releasing more antioxidants including phenolic compounds to the solvents. The factor time when increased allowed the solutes to be in contact with solvent for longer facilitating higher diffusion of the target compounds (Ghafoor et al., 2009). The highest total phenolic content (9.62 g GAE/100 g DW) was observed in the extracts obtained at 61 µm amplitude coupled with 35 °C after 10 min of treatment among the treatments generated by RSM. This value was 98.39% higher than that of solid/liquid extract.

**Effect of thermosonication on ferric reducing antioxidant power**

All three factors had significant (p<0.05 to 0.0001) linear effect on enhancing the FRAP values of the extracts of marjoram, oregano, rosemary and sage. Additionally temperature and time showed quadratic and interaction effect respectively in marjoram. On the other hand, in rosemary both amplitude and time had quadratic and interaction effects along with the linear effects on the FRAP values of its extract (Table). Among the factors, amplitude of ultrasonication showed the highest effect followed by temperature and time. The effect of temperature and time was in agreement with the finding of Ghafoor et al. (2009) in analysing the antioxidant activity of grape extracts obtained using fixed level of ultrasound. Among the ultrasonication treatments used, the amplitude level of 61 µm with the temperature of 35 °C after 10 min showed the highest FRAP values in the herbs examined ranging from 12.02 g Trolox/100 g DW in oregano to 19.56 g Trolox/100 g
DW in rosemary). This treatment in marjoram increased the antioxidant activity as measured by FRAP by 89.76% compared to conventional solid/liquid extracts (9.00 g Trolox/100 g DW). Similar results were observed in the other herbs used in the current study. In fact, all the ultrasonication treated extracts showed significantly (p<0.05) higher FRAP values than that of solid/liquid extracts. When the ultrasonication amplitude was increased from the lowest level (24.4 µm) to the highest level (61 µm), the FRAP value showed an increase of 26.98%. In spices, antioxidant activity is related to their total phenol content. The high values of pearsons correlation coefficient (r=0.90) reflects the importance of phenols for antioxidant capacity of the herbs examined.

**Effect of thermosonication on different polyphenols**

The principal polyphenol identified in the extracts of marjoram, oregano, rosemary and sage was rosmarinic acid (Hossain *et al.*., 2010). All the factors (amplitude, temperature and time) had significant (p<0.05) positive linear effects on the rosmarinic acid content (Figure 2A) of marjoram, rosemary and sage extracts. The effect of amplitude was higher than that of other factors. This result was in agreement with the finding of Albu *et al.* (25). In case of oregano, the effect of time in extracting rosmarinic acid was not significant (p<0.05) at both linear and quadratic levels. The temperature had a significant (p<0.05) quadratic effect on all the spices examined except rosemary where time showed the quadratic effect. On the other hand, the dominant factor amplitude had quadratic effect only in sage. The interaction effect between temperature and time was significant (P<0.05) in marjoram, rosemary and sage. In sage, temperature had additional interaction effect with amplitude during extraction of rosmarinic acid content. Higher levels of time
and temperature could have increased further the extraction of antioxidant polyphenols. But this would have increased the cost of extraction and an environmentally friendly extraction method requires minimal extraction time and temperature (Ghafoor et al., 2009). Therefore, in the present study, time and temperature range was kept low. The lowest value of rosmarinic acid content (8.42 mg/g DW) was observed in oregano at an amplitude level of 24.4 µm treated for 5 min at 25 °C. With the increase of amplitude of ultrasonication the rosmarinic acid content of the extracts increased gradually. At the highest amplitude and temperature used in the present study, the content of rosmarinic acid was 11.65 mg/g DW which was approximately two times higher than that of solid/liquid extracts (5.65 mg/g DW) (Figure 3). Similar results were observed in other herbs tested. Increase of rosmarinic acid extraction from dried rosemary with the increase of ultrasonication amplitude has also been reported by Paniwnyk et al. (2009). The other hydroxycinnamic acid derivative investigated, caffeic acid, was affected predominantly by temperature in marjoram at quadratic level showing higher extractions at two ends of the temperature range used (Figure 2B). Temperature also showed significant (p<0.05) positive effect in interaction with amplitude. In rosemary, oregano and sage, amplitude was the dominant factor affecting the caffeic acid content of the extracts of mentioned herbs. The major flavonoids of Lamiaceae spices are luteolin-7-O-glucoside and apigenin-7-O-glucoside. Both these flavonoids showed significant (p<0.05) increase with the increase of amplitude and temperature in all the herbs used, while time did not have any significant effect (Figure 2C,D) in marjoram and rosemary. In the case of luteolin-7-O-glucoside, temperature also had quadratic and interaction effects with amplitude. Amplitude and temperature played the dominant role in extracting flavonoids. The
antioxidant volatile polyphenols carnosic acid and carnosol showed significant (p<0.05) increases with the increase of amplitude (Figure 2E,F). Temperature also had significant (p<0.05) effect on carnosic acid and carnosol content of the herbs extracts except marjoram extracts. However, time had less pronounced effect on these two volatiles. The effect of time on carnosol content of marjoram, oregano and rosemary extracts was not significant (p<0.05). Carnosic acid content of the herbs was significantly affected by time except in marjoram. Paniwnyk et al. (2009) also found an increase of extraction of carnosic acid from rosemary with increased amplitude of ultrasonication.

Optimization and model validation
The RSM guided optimization demonstrated that the optimum treatments for maximizing the TP values of the extracts of the herbs used were in the range of 60.32 to 61 µm (amplitude), 34.08 to 35 °C (temperature) and 9.64 to 14.80 min (time). The optimum treatments for getting maximum FRAP values were identified in the range of 54.34 to 61 µm (amplitude), 32.79 to 35 °C (temperature) and 11.18 to 14.89 min (time). The antioxidant polyphenols namely rosmarinic acid, luteolin-7-O-glucoside, apigenin-7-O-glucoside, caffeic acid, carnosic acid and carnosol had optimum extraction conditions at the amplitude level from 55.42 µm to 61 µm with a combination of time and temperature ranging from 9 to 15 min and 30.26 to 35 °C respectively. In all the herbs examined, the optimal extraction condition for all the parameters combined was 61 µm, 35 °C and 15 min. The predicted values at the optimal conditions were in close agreement with experimental values (Table 1) and were found to be not significantly different at p > 0.05 using a paired t-test. In addition variations between the predicted and experimental values
obtained for total antioxidant activity by FRAP assay, TP content and antioxidant polyphenols were within acceptable error range as depicted by average mean deviation (E%, Table 1); therefore, the predictive performance of the established model may be considered acceptable. These values were significantly (p<0.05) higher than those of solid/liquid extracts (Table 1).

**Model fitting**

The analysis of variance showed that the R-squared statistic of all the parameters was in the range of 0.710 to 0.996 indicating high representation of the variability of the parameters by the models. The quadratic polynomial models generated were highly significant with p-value ranging from 0.03 to 0.0001. The lack of fit statistics of all the parameters were not significant (p>0.05) and high degree of F-value (range 4.48-130.6) further strengthened the reliability of the models (Table 1-4). The predicted values obtained by the quadratic polynomial equations showed strong correlation with actual experimental values with pearsons correlation coefficients (r) from 0.88 to 0.98.

**ACKNOWLEDGEMENT**

We would like to thank AllinAll Ingredients Ltd, Dublin 12 for providing spice samples.

**LITERATURE CITED**


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Figure captions

Figure 1. Contour plots showing the effect of amplitude and temperature on total phenol content (A) and antioxidant activity as measured by FRAP (B) at treatment time of 10 min.

Figure 2. Contour plots showing the effect of amplitude and temperature on the extraction of rosmarinic acid (A), caffeic acid (B), luteolin-7-O-glucoside (C), apigenin-7-O-glucoside (D), carnosic acid (E) and carnosol (F) at treatment time of 10 min.

Figure 3. HPLC chromatograms of UAE extracts of marjoram obtained at 61 µm, 35 ºC and 10 min (A), in comparison to solid/liquid extracts (B) showing the changes in peaks of different polyphenols (1=caffeic acid, 2=luteolin-7-O-glucoside, 3=apigenin-7-O-glucoside, 4=rosmarinic acid, 5=carnosol and 6=carnosic acid).
Table 1. Predicted and experimental values of the parameters tested at optimal UAE condition in comparison to the conventional solid/liquid extraction values and average mean deviation between predicted and experimental values of optimal UAEa.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum UAE condition for all the parameters combined</th>
<th>Predicted values at optimal UAE</th>
<th>Desirability</th>
<th>Experimental values at optimal UAE</th>
<th>E%</th>
<th>Solid/liquid extraction values</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (g GAE/ 100 g DW)</td>
<td>61 µm, 35 °C and 15 min</td>
<td>9.90</td>
<td>0.984</td>
<td>9.51±0.10</td>
<td>1.34</td>
<td>4.85±0.05</td>
</tr>
<tr>
<td>FRAP (g Trolox/100 g DW)</td>
<td></td>
<td>18.56</td>
<td></td>
<td>18.96±0.19</td>
<td>0.70</td>
<td>9.00±0.17</td>
</tr>
<tr>
<td>Rosmarinic acid (mg/g DW)</td>
<td></td>
<td>24.53</td>
<td></td>
<td>24.86±0.45</td>
<td>0.45</td>
<td>12.08±0.03</td>
</tr>
<tr>
<td>Luteolin-7-O-glucoside (mg/g DW)</td>
<td></td>
<td>5.38</td>
<td></td>
<td>5.27±0.14</td>
<td>0.65</td>
<td>2.69±0.02</td>
</tr>
<tr>
<td>Apigenin-7-O-glucoside (mg/g DW)</td>
<td></td>
<td>1.54</td>
<td></td>
<td>1.60±0.13</td>
<td>1.31</td>
<td>0.86±0.01</td>
</tr>
<tr>
<td>Caffeic acid (mg/g DW)</td>
<td></td>
<td>0.15</td>
<td></td>
<td>0.14±0.01</td>
<td>1.55</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>Carnosic acid (mg/g DW)</td>
<td></td>
<td>10.25</td>
<td></td>
<td>10.63±0.26</td>
<td>1.20</td>
<td>3.56±0.15</td>
</tr>
<tr>
<td>Carnosol (mg/g DW)</td>
<td></td>
<td>1.72</td>
<td></td>
<td>1.81±0.16</td>
<td>1.36</td>
<td>0.72±0.02</td>
</tr>
</tbody>
</table>

a Data are expressed as means ± SD (n=3)
Table 2. Analysis of the variance of the regression coefficients of the fitted polynomial quadratic equation for TP (g GAE/ 100 g DW), FRAP (g Trolox/100 g DW) and different polyphenols (mg/g DW).

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>TP</th>
<th>FRAP</th>
<th>Rosmarinic acid</th>
<th>Luteolin-7-O-glucoside</th>
<th>Apigenin-7-O-glucoside</th>
<th>Caffeic acid</th>
<th>Carnosic acid</th>
<th>Carnosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>β0 (Intercept)</td>
<td>+12.11</td>
<td>+21.36</td>
<td>+19.43</td>
<td>+5.22</td>
<td>+0.44</td>
<td>+0.29</td>
<td>+7.72</td>
<td>+0.96</td>
</tr>
<tr>
<td>Linear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β1 (Amplitude)</td>
<td>-3.28x10^-2b</td>
<td>-4.79x10^-2d</td>
<td>+6.99x10^-2d</td>
<td>+1.08x10^-2d</td>
<td>+8.04x10^-3b</td>
<td>-1.73x10^-3ns</td>
<td>+1.1x10^-3d</td>
<td>+7.45x10^-3c</td>
</tr>
<tr>
<td>β2 (Temperature)</td>
<td>-3.29x10^-1b</td>
<td>-4.49x10^-1d</td>
<td>-6.27x10^-2d</td>
<td>-1.10x10^-1a</td>
<td>+1.23x10^-2b</td>
<td>-1.05x10^-2ns</td>
<td>-8.6x10^-2ns</td>
<td>+5.44x10^-3ns</td>
</tr>
<tr>
<td>β3 (Time)</td>
<td>-9.93x10^-2a</td>
<td>-5.17x10^-1c</td>
<td>+1.9x10^-1c</td>
<td>-9.67x10^-5ns</td>
<td>+1.20x10^-2ns</td>
<td>-1.45x10^-5ns</td>
<td>-9.93x10^-5ns</td>
<td>+6.84x10^-5ns</td>
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<td>Quadratic</td>
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<td></td>
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<td></td>
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<tr>
<td>β11</td>
<td>+1.13x10^-4ns</td>
<td>+3.12x10^-4ns</td>
<td>-3.97x10^-4ns</td>
<td>-2.75x10^-4a</td>
<td>-1.20x10^-2ns</td>
<td>-1.27x10^-3a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β22</td>
<td>+4.83x10^-3b</td>
<td>+7.39x10^-3c</td>
<td>+3.04x10^-3b</td>
<td>+1.22x10^-3b</td>
<td>-1.50x10^-3b</td>
<td>+9.11x10^-4a</td>
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<td>+2.09x10^-4ns</td>
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<td>β12</td>
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<td>+1.13x10^-3c</td>
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<td>+1.19x10^-2c</td>
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<td>+6.92x10^-4ns</td>
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<td>-1.2x10^-4ns</td>
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<td>5.11</td>
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<td>0.183</td>
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<td>F-value</td>
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*ns Not significant
*a significant at p≤0.05
*b significant at p≤0.01
*c significant at p≤0.001
*d significant at p≤0.0001
Figure 1

A

TP (g GAE/100 g DW)

B

FRAP (g Trolox/100 g DW)
Figure 2

A  Rosmarinic acid (mg/g DW)

B  Caffeic acid (mg/g DW)

C  Luteolin-7-O-glucoside (mg/g DW)

D  Apigenin-7-O-glucoside (mg/g DW)

E  Camosic acid (mg/g DW)

F  Camosol (mg/g DW)
Figure 3