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Optimization of Ultrasound Assisted Extraction of Antioxidant Compounds from Marjoram (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 (R^2) ranging from 0.710 to 0.989.

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

70   extraction, microwave-assisted extraction, and solvent extraction (*14, 15*). Among these,

71   ultrasound-assisted extraction (UAE) offers an inexpensive, environmentally friendly,

72   less time consuming and efficient alternative to conventional extraction techniques. The

73   enhancement in extraction obtained by using ultrasound is mainly attributed to the effect

74   of acoustic cavitations produced in the solvent by the passage of an ultrasound wave (*16,*

75   *17*). Ultrasound also offers a mechanical effect allowing greater penetration of solvent

76   into the sample matrix, increasing the contact surface area between the solid and liquid

77   phase, and as a result, the solute quickly diffuses from the solid phase to the solvent (*18,*

78   *19*).

79   In this study, UAE parameters such as extraction temperature, extraction time and

80   amplitude of ultrasound were optimized using response surface methodology (RSM), by

81   employing a Box-Behnken design to maximize extraction of antioxidant polyphenolic

82   compounds from marjoram.

83

84   **MATERIALS AND METHODS**

85   **Samples and reagents.** Dried and ground marjoram leaf was provided by AllinAll

86   Ingredients Limited, Dublin 12. The country of origin of the spices was Turkey. The

87   plants were grown in sunny and well drained land with annual rainfall of around 15

88   inches. As per the product specifications the samples were air dried at ambient

89   temperature (~ 23 °C) after heat treatment (steam sterilization at 120 °C for 30 sec).

90   Folin-Ciocalteu Reagent, gallic acid, sodium acetate anhydrous, ferric chloride

91   hexahydrate, 2,4,6-Tri(2-pyridyl)-s-triazine, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-

92 carboxylic acid, sodium carbonate, caffeic acid, rosmarinic acid, luteolin-7-*O*-glucoside,

93 apigenin-7-*O*-glucoside, carnosic acid and carnosol were purchased from Sigma-Aldrich.

94

95 **Sonication treatment.** A 1500W ultrasonic processor (VC 1500, Sonics and Materials

96 Inc., Newtown, USA) with a 19 mm diameter probe was used for sonication. Samples

97 were processed at a constant frequency of 20 kHz. The energy input was controlled by

98 setting the amplitude of the sonicator probe. Extrinsic parameters of amplitude (24.4–

99 61.0 µm), temperature (15–35 °C) and processing time (5–15 min) were varied with pulse

100 durations of 5 s on and 5 s off. Dried leaf particles of marjoram (1 g) were placed in a 50

101 mL jacketed vessel through which water was circulated at 15±0.5, 25± 0.5 and 35±0.5 °C

102 with a flow rate of 0.5 L/min. Sonication at the desired amplitude level was started once

103 the set temperature was reached. The ultrasound probe was submerged to a depth of 25

104 mm in the sample. All treatments were carried out in triplicate.

105

106 **Conventional solid/liquid extraction.** Solid/liquid extractions were carried out

107 according to the method of Shan et al. (*3*) with slight modifications. Briefly, dried and

108 ground samples (0.5 g) were homogenized for 1 min at 24,000 rpm using an Ultra-Turrax

109 T-25 Tissue homogenizer (Janke & Kunkel, IKA®-Labortechnik, Saufen, Germany) in 25

110 mL of 80% methanol at room temperature (~23 °C). The homogenized sample

111 suspension was shaken for 3 hours with a V400 Multitude Vortexer (Alpha laboratories,

112 North York, Canada) at 1,500 rpm at room temperature ($\cong$ 25°C). The sample suspension

113 was then centrifuged for 15 min at 2,000 g (MSE Mistral 3000i, Sanyo Gallenkamp,

114 Leicestershire, UK) and immediately filtered through 0.45 µm polytetrafluoethylene

115 (PTFE) filters. The extracts were kept at -20 °C until subsequent analysis. The

116 experiment was performed in two batches which included three replications of each

117 sample.

118

119 **Determination of total phenol (TP).** The total phenolic content was determined using

120 Folin-Ciocalteu Reagent (FCR) as described by Singelton et al. (*20*). The experiment was

121 performed in two batches which included three replications of each sample and standard.

122 Methanolic gallic acid solutions (10-400 mg/L) were used as standards. In each replicate,

123 100 µL of the appropriately diluted sample extract, 100 µL methanol, 100 µL FCR and

124 finally 700 µL Na_2CO_3 (20%) were added together and vortexed. The mixture was

125 incubated for 20 min in the dark and room temperature. After incubation the mixture was

126 centrifuged at 13,000 rpm for 3 min. The absorbance of the supernatant was measured at

127 735 nm by spectrophotometer. The total phenolic content was expressed as gallic acid

128 equivalent (GAE)/100 g dry weight (DW) of the sample.

129

130 **Ferric ion reducing antioxidant power (FRAP) assay.** The FRAP assay was carried

131 out as described by Stratil et al. (*21*) with slight modifications. The FRAP reagent was

132 prepared by mixing 38 mM sodium acetate anhydrous in distilled water pH 3.6, 20 mM

133 $FeCl_3.6H_2O$ in distilled water and 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40

134 mM HCl in a proportion of 10:1:1. This reagent was freshly prepared before each

135 experiment. To each sample 100 µL of appropriately diluted sample extract and 900 µL

136 of FRAP reagent was added and the mixture was incubated at 37 °C for 40 min in the

137 dark. In the case of the blank 100 µL of methanol was added to 900 µL of FRAP reagent.

138   The absorbance of the resulting solution was measured at 593 nm by spectrophotometer.

139   Trolox   (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic   acid)   (a   synthetic

140   antioxidant) at concentrations from 0.1 mM-0.4 mM was used as a reference antioxidant

141   standard. FRAP values were expressed as g Trolox/100 g DW of the sample.

142

143   **HPLC analysis of the extracts.** Reversed phase high performance liquid

144   chromatography (RP-HPLC) of the filtered sample extracts were carried out according to

145   the method of Tsao and Yang (*22*). The chromatographic system (Shimadzu-Model no

146   SPD-M10A VP, Mason Technology, Dublin 8, Ireland) consisted of a pump, a vacuum

147   degasser, a Diode-Array Detector and was controlled through EZ Start 7.3 software

148   (Shimadzu) at 37 °C. An Agilent C18 column (15 cm × 4.6 cm, 5 μm, Agilent

149   Technologies., USA) was utilised with a binary mobile phase of 6 % acetic acid in 2 mM

150   sodium acetate (final pH 2.55, v/v, solvent A) and acetonitrile (solvent B). Solvent A was

151   prepared first by making 2 mM sodium acetate water solution, which was then mixed

152   with acetic acid at a ratio of 94:6 by volume. All solvents were filtered through a 0.45 μm

153   membrane filter prior to analysis. The flow rate was kept constant at 1.0 mL/min for a

154   total run time of 80 min. The following gradient program was carried out: 0-15% B in 45

155   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

156   min. The injection volume for all the samples was 10 μL. All the standards for

157   quantification purposes were dissolved in methanol. The detection wavelength of 280 nm

158   was used for the detection of carnosol and carnosic acid. Rosmarinic acid, caffeic acid

159   and apigenin-7-*O*-glucoside were detected at 320 nm while luteilon-7-*O*-glucoside was

160   detected at 360 nm. Identification of the compounds was achieved by comparing their

161 retention times and UV-Vis spectra with those of authenticated standards by using the

162 inline DAD with a 3D feature. Results are expressed as mean values of three assays for

163 each replicated experiment.

164

165 **Experimental design and data analysis.** Polynomial regression equations were

166 developed to describe the effects of the 3 independent processing parameters; ultrasound

167 amplitude (X_1, μm), extraction temperature (X_2, °C) and processing time (X_3, min) on

168 total phenol (TP), antioxidant activity as measured by FRAP and different polyphenolic

169 compounds such as rosmarinic acid, caffeic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-

170 glucoside, carnosol and carnosic acid. Independent variables of amplitude level (X_1)

171 (24.4, 42.7, and 61 μm), temperature (X_2) (15, 25, 35 °C), and processing time (X_3) (5, 10

172 and 15 min) were varied to investigate the effects on dependent variables mentioned

173 above. The general form of the quadratic polynomial model regression equation

174 employed in this study is presented in Eq. 1. By using this equation, linear (X_1, X_2, X_3),

175 quadratic (X_1^2, X_2^2, X_3^2) and interactive (X_1X_2, X_1X_3, X_2X_3) effects of independent

176 variables, temperature (X_1), amplitude level (X_2), and time (X_3) on dependent variable

177 (Y) were determined.

178 $$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \ \ldots\ldots\ldots\ldots..(Eq.\ 1)$$

179 Where Y is the predicted response; β_0 the constant (intercept); β_i the linear coefficient; β_{ii}

180 the quadratic coefficient and β_{ij} is the cross product coefficient. X_i and X_j are independent

181 variables. The response surface regression was used to analyze the experimental data

182 using Design Expert Version 7.1.3 software (Stat-Ease, Inc., Minneapolis, MN). Two

183 dimensional contour plots were developed while holding a variable constant in the second

184 order polynomial models. All processing trials were conducted in triplicate.

185

186 **Model validation.** The predictive performance of the developed models describing the

187 combined effect amplitude (X_1), temperature (X_2) and time (X_3) on independent variables

188 (FRAP, TP, rosmarinic acid, caffeic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-

189 glucoside, carnosol and carnosic acid) of marjoram were validated with optimal

190 extraction conditions as predicted by the design.

191 The criterion used to characterize the fitting efficiency of the data to the model was the

192 multiple correlation coefficients (R^2) and their average mean deviation (E, Eq. 2).

193
$$E(\%) = \frac{1}{n_e} \sum_{i=1}^{n} \left\| \frac{V_E - V_P}{V_E} \right\| \times 100 \qquad \ldots\ldots\ldots\ldots(Eq.\ 2)$$

194 where, n_e is the number of experimental data, V_E is the experimental value and V_P is the

195 predicted value.

196

197 **RESULTS AND DISCUSSION**

198 **Effect of thermosonication on total phenol content**

199 Figure 1A presents the contour plot showing the effect of three different parameters of

200 UAE such as ultrasound amplitude, temperature, and time on the total phenol content of

201 marjoram, oregano, rosemary and sage extracts. All the three factors had significant

202 (p<0.05) positive effect on the total phenol content of these extracts. Among the factors,

203 amplitude showed the highest effect followed by temperature and time except in oregano

204 where the order of effect of the parameters was amplitude > time > temperature. An

205 increase in temperature increases target compound solubility, solvent diffusion rate and

206   mass transfer, while solvent viscosity and surface tension decrease (Hossain *et al*., 2010).

207   Reduced viscosity and surface tension facilitates the solvent to access deeper into sample

208   matrix which enhances extraction efficiency by exposing more surface area of the sample

209   to solvents used. Higher amplitude of ultrasound could have damaged more cell walls

210   releasing more antioxidants including phenolic compounds to the solvents. The factor

211   time when increased allowed the solutes to be in contact with solvent for longer

212   facilitating higher diffusion of the target compounds (Ghafoor *et al*., 2009). The highest

213   total phenolic content (9.62 g GAE/100 g DW) was observed in the extracts obtained at

214   61 µm amplitude coupled with 35 °C after 10 min of treatment among the treatments

215   generated by RSM. This value was 98.39% higher than that of solid/liquid extract.

216

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

218   All three factors had significant ($p<0.05$ to 0.0001) linear effect on enhancing the FRAP

219   values of the extracts of marjoram, oregano, rosemary and sage. Additionally temperature

220   and time showed quadratic and interaction effect respectively in marjoram. On the other

221   hand, in rosemary both amplitude and time had quadratic and interaction effects along

222   with the linear effects on the FRAP values of its extract (Table). Among the factors,

223   amplitude of ultrasonication showed the highest effect followed by temperature and time.

224   The effect of temperature and time was in agreement with the finding of Ghafoor *et al*.

225   (2009) in analysing the antioxidant activity of grape extracts obtained using fixed level of

226   ultrasound. Among the ultrasonication treatments used, the amplitude level of 61 µm

227   with the temperature of 35 °C after 10 min showed the highest FRAP values in the herbs

228   examined ranging from 12.02 g Trolox/100 g DW in oregano to 19.56 g Trolox/100 g

229   DW in rosemary). This treatment in marjoram increased the antioxidant activity as

230   measured by FRAP by 89.76% compared to conventional solid/liquid extracts (9.00 g

231   Trolox/100 g DW). Similar results were observed in the other herbs used in the current

232   study. In fact, all the ultrasonication treated extracts showed significantly ($p<0.05$) higher

233   FRAP values than that of solid/liquid extracts. When the ultrasonication amplitude was

234   increased from the lowest level (24.4 µm) to the highest level (61 µm), the FRAP value

235   showed an increase of 26.98%. In spices, antioxidant activity is related to their total

236   phenol content. The high values of pearsons correlation coefficient (r=0.90) reflects the

237   importance of phenols for antioxidant capacity of the herbs examined.

238

239   **Effect of thermosonication on different polyphenols**

240   The principal polyphenol identified in the extracts of marjoram, oregano, rosemary and

241   sage was rosmarinic acid (Hossain *et al*., 2010). All the factors (amplitude, temperature

242   and time) had significant ($p<0.05$) positive linear effects on the rosmarinic acid content

243   (Figure 2A) of marjoram, rosemary and sage extracts. The effect of amplitude was higher

244   than that of other factors. This result was in agreement with the finding of Albu *et al*.

245   (*25*). In case of oregano, the effect of time in extracting rosmarinic acid was not

246   significant ($p<0.05$) at both linear and quadratic levels. The temperature had a significant

247   ($p<0.05$) quadratic effect on all the spices examined except rosemary where time showed

248   the quadratic effect. On the other hand, the dominant factor amplitude had quadratic

249   effect only in sage. The interaction effect between temperature and time was significant

250   ($P<0.05$) in marjoram, rosemary and sage. In sage, temperature had additional interaction

251   effect with amplitude during extraction of rosmarinic acid content. Higher levels of time

252   and temperature could have increased further the extraction of antioxidant polyphenols.

253   But this would have increased the cost of extraction and an environmentally friendly

254   extraction method requires minimal extraction time and temperature (Ghafoor *et al.*,

255   2009). Therefore, in the present study, time and temperature range was kept low. The

256   lowest value of rosmarinic acid content (8.42 mg/g DW) was observed in oregano at an

257   amplitude level of 24.4 µm treated for 5 min at 25 °C. With the increase of amplitude of

258   ultrasonication the rosmarinic acid content of the extracts increased gradually. At the

259   highest amplitude and temperature used in the present study, the content of rosmarinic

260   acid was 11.65 mg/g DW which was approximately two times higher than that of

261   solid/liquid extracts (5.65 mg/g DW) (Figure 3). Similar results were observed in other

262   herbs tested. Increase of rosmarinic acid extraction from dried rosemary with the increase

263   of ultrasonication amplitude has also been reported by Paniwnyk *et al.* (2009). The other

264   hydroxycinnamic acid derivative investigated, caffeic acid, was affected predominantly

265   by temperature in marjoram at quadratic level showing higher extractions at two ends of

266   the temperature range used (Figure 2B). Temperature also showed significant (p<0.05)

267   positive effect in interaction with amplitude. In rosemary, oregano and sage, amplitude

268   was the dominant factor affecting the caffeic acid content of the extracts of mentioned

269   herbs. The major flavonoids of Lamiaceae spices are luteolin-7-*O*-glucoside and

270   apigenin-7-*O*-glucoside. Both these flavonoids showed significant (p<0.05) increase with

271   the increase of amplitude and temperature in all the herbs used, while time did not have

272   any significant effect (Figure 2C,D) in marjoram and rosemary. In the case of luteolin-7-

273   *O*-glucoside, temperature also had quadratic and interaction effects with amplitude.

274   Amplitude and temperature played the dominant role in extracting flavonoids. The

275   antioxidant volatile polyphenols carnosic acid and carnosol showed significant (p<0.05)

276   increases with the increase of amplitude (Figure 2E,F). Temperature also had significant

277   (p<0.05) effect on carnosic acid and carnosol content of the herbs extracts except

278   marjoram extracts. However, time had less pronounced effect on these two volatiles. The

279   effect of time on carnosol content of marjoram, oregano and rosemary extracts was not

280   significant (p<0.05). Carnosic acid content of the herbs was significantly affected by time

281   except in marjoram. Paniwnyk et al. (2009) also found an increase of extraction of

282   carnosic acid from rosemary with increased amplitude of ultrasonication.

283

284   **Optimization and model validation**

285   The RSM guided optimization demonstrated that the optimum treatments for maximizing

286   the TP values of the extracts of the herbs used were in the range of 60.32 to 61 µm

287   (amplitude), 34.08 to 35 °C (temperature) and 9.64 to 14.80 min (time). The optimum

288   treatments for getting maximum FRAP values were identified in the range of 54.34 to 61

289   µm (amplitude), 32.79 to 35 °C (temperature) and 11.18 to 14.89 min (time). The

290   antioxidant polyphenols namely rosmarinic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-

291   glucosdie, caffeic acid, carnosic acid and carnosol had optimum extraction conditions at

292   the amplitude level from 55.42 µm to 61 µm with a combination of time and temperature

293   ranging from 9 to 15 min and 30.26 to 35 °C respectively. In all the herbs examined, the

294   optimal extraction condition for all the parameters combined was 61 µm, 35 °C and 15

295   min. The predicted values at the optimal conditions were in close agreement with

296   experimental values (Table 1) and were found to be not significantly different at p > 0.05

297   using a paired t-test. In addition variations between the predicted and experimental values

298   obtained for total antioxidant activity by FRAP assay, TP content and antioxidant

299   polyphenols were within acceptable error range as depicted by average mean deviation

300   (E%, Table 1); therefore, the predictive performance of the established model may be

301   considered acceptable. These values were significantly ($p<0.05$) higher than those of

302   solid/liquid extracts (Table 1).

303

304   **Model fitting**

305   The analysis of variance showed that the R-squared statistic of all the parameters was in

306   the range of 0.710 to 0.996 indicating high representation of the variability of the

307   parameters by the models. The quadratic polynomial models generated were highly

308   significant with p-value ranging from 0.03 to 0.0001. The lack of fit statistics of all the

309   parameters were not significant ($p>0.05$) and high degree of F-value (range 4.48-130.6)

310   further strengthened the reliability of the models (Table 1-4). The predicted values

311   obtained by the quadratic polynomial equations showed strong correlation with actual

312   experimental values with pearsons correlation coefficients (r) from 0.88 to 0.98.

313

316

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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.

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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.

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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).

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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 UAE[a].

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

[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).

Coefficients	TP	FRAP	Rosmarinic acid	Luteolin-7-O-glucoside	Apigenin-7-O-glucoside	Caffeic acid	Carnosic acid	Carnosol
β_0 (Intercept)	+12.11	+21.36	+19.43	+5.22	+0.44	+0.29	+7.72	+0.96
Linear								
β_1 (Amplitude)	-3.28×10^{-2b}	-4.79×10^{-2d}	$+6.99\times10^{-2d}$	$+1.08\times10^{-2d}$	$+8.04\times10^{-3b}$	-1.73×10^{-3ns}	$+1.1\times10^{-1d}$	$+7.45\times10^{-3c}$
β_2 (Temperature)	-3.29×10^{-1b}	-4.49×10^{-1d}	-6.27×10^{-2d}	-1.10×10^{-1a}	$+1.23\times10^{-2b}$	-1.05×10^{-2ns}	-8.6×10^{-2ns}	$+5.44\times10^{-3ns}$
β_3 (Time)	-9.93×10^{-2a}	-5.17×10^{-1c}	$+1.9\times10^{-1c}$	-9.67×10^{-3ns}	$+1.20\times10^{-2ns}$	-1.45×10^{-3ns}	-9.93×10^{-3ns}	$+6.84\times10^{-3ns}$
Quadratic								
β_{11}	$+1.13\times10^{-4ns}$	$+3.12\times10^{-4ns}$	-3.97×10^{-4ns}	-2.75×10^{-4a}	-	$+1.20\times10^{-5ns}$	-1.27×10^{-3d}	-
β_{22}	$+4.83\times10^{-3b}$	$+7.39\times10^{-3c}$	$+3.04\times10^{-3b}$	$+1.22\times10^{-3b}$	-	$+1.50\times10^{-4b}$	$+9.11\times10^{-4a}$	-
β_{33}	-7.99×10^{-4ns}	$+2.38\times10^{-3ns}$	$+8.27\times10^{-4ns}$	-1.27×10^{-3ns}	-	-7.20×10^{-5ns}	$+2.09\times10^{-4ns}$	-
Cross product								
β_{12}	$+1.55\times10^{-3a}$	$+5.46\times10^{-4ns}$	$+2.27\times10^{-4ns}$	$+1.13\times10^{-3c}$	-	$+3.72\times10^{-5a}$	$+1.1\times10^{-3b}$	-
β_{13}	$+3.25\times10^{-4ns}$	$+5.95\times10^{-3b}$	-3.54×10^{-4ns}	$+5.91\times10^{-4ns}$	-	$+6.63\times10^{-5ns}$	$+1.8\times10^{-4ns}$	-
β_{23}	$+5.79\times10^{-3a}$	$+1.19\times10^{-2c}$	-4.61×10^{-4a}	$+6.92\times10^{-4ns}$	-	$+1.19\times10^{-4ns}$	-1.2×10^{-4ns}	-
R^2	0.917	0.982	0.975	0.986	0.721	0.874	0.989	0.71
CV	2.81	1.40	0.74	1.22	8.04	4.62	0.79	5.11
p	0.0048	0.0001	0.0001	0.0001	0.0007	0.0183	0.0001	0.0009
Lack of fit	0.073	0.067	0.183	0.147	0.137	0.210	0.129	0.149
F-value	8.62	43.06	30.34	55.27	11.23	5.41	73.54	10.46

[ns] Not significant
[a] significant at $p\leq0.05$
[b] significant at $p\leq0.01$
[c] significant at $p\leq0.001$
[d] significant at $p\leq0.0001$

Figure 1

A
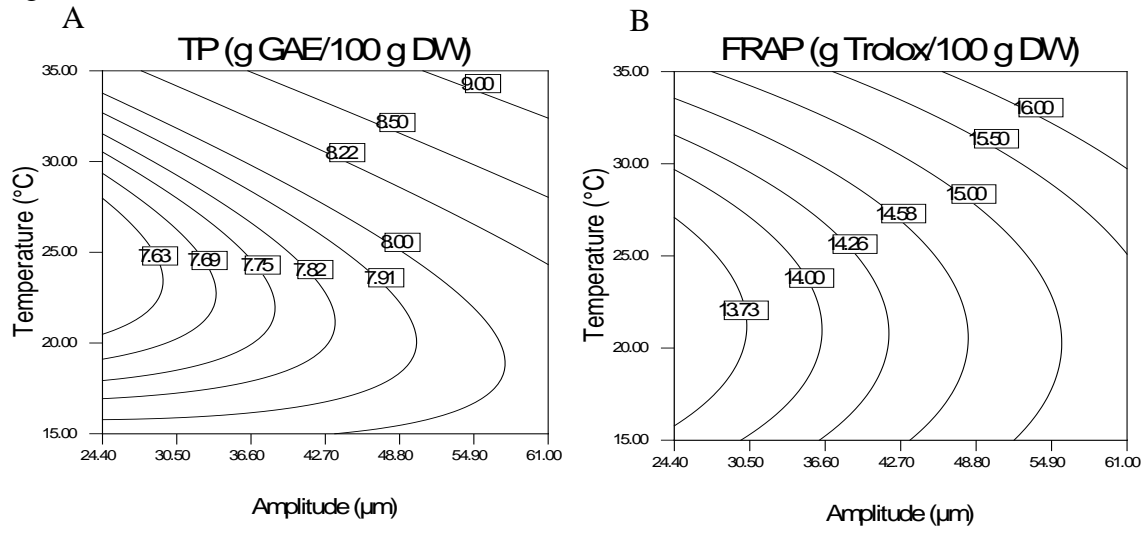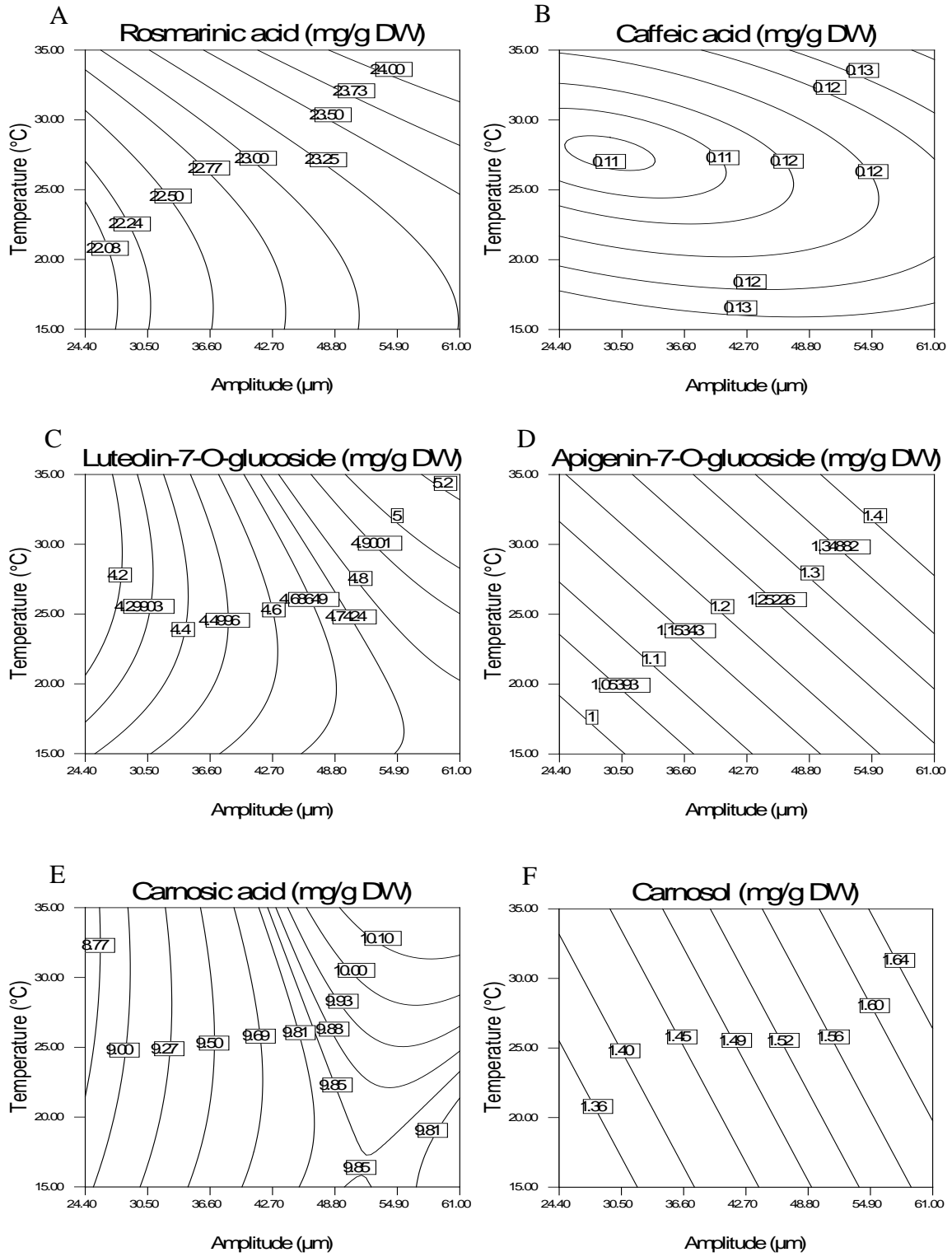
TP (g GAE/100 g DW)

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
Carnosic acid (mg/g DW)

F
Carnosol (mg/g DW)

Figure 3