

2012

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

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Recommended Citation

Hossain, M., Brunton, N., Patras, A., Tiwari, B., O'Donnell, C., Martin-Diana, A., Barry-Ryan, C.: Optimization of ultrasound assisted extraction of antioxidant compounds from marjoram (*Origanum majorana* L.) using response surface methodology. *Ultrasonics Sonochemistry*, Vol. 19, (3), 2012, pp.582-590.

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Funder: Irish Department of Agriculture, Fisheries and Food under the Food Institutional Research Measure

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1 **Optimization of ultrasound assisted extraction of antioxidant compounds from**
2 **Marjoram (*Origanum majorana* L.) using response surface methodology**

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24 **ABSTRACT**

25 The present study optimized the ultrasound assisted extraction (UAE) conditions to
26 maximize the antioxidant activity [Ferric ion Reducing Antioxidant Power (FRAP)], total
27 phenol content (TP) and content of individual polyphenols of the extracts from four
28 Lamiaceae herbs namely marjoram, oregano, rosemary and sage. Optimal conditions with
29 regard to amplitude of sonication (24.4–61.0 μm) and extraction temperature (15-35 $^{\circ}\text{C}$)
30 and time (5-15 min) were identified using response surface methodology (RSM). The
31 results showed that the combined treatment of 61 μm , 35 $^{\circ}\text{C}$ and 15 min was optimal for
32 maximizing TP, FRAP, rosmarinic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside,
33 caffeic acid, carnosic acid and carnosol values of the extracts. The predicted values from
34 the quadratic polynomial equation were in close agreement with the actual experimental
35 values with low average mean deviation (E%) ranging from 0.45 to 1.55 %. The
36 extraction yields of the optimal UAE were significantly ($p < 0.05$) higher than solid/liquid
37 extracts. Predicted models were highly significant ($p < 0.05$) for all the parameters studied
38 with high regression coefficients (R^2) ranging from 0.710 to 0.989.

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40 **KEYWORDS:** Antioxidant, spice, ultrasound assisted extraction, total phenols, RSM

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47 **INTRODUCTION**

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

62 Oxidation of polyunsaturated fatty acids not only lowers the nutritional value of food
63 (11), but is also associated with cell membrane damage, aging, heart disease and cancer
64 in living organisms (12). Therefore the addition of natural antioxidants to food products
65 has become popular as a means of increasing shelf life and to reduce wastage and
66 nutritional losses by inhibiting and delaying oxidation (13). However, an efficient
67 extraction technique is required in order to harvest the benefits of natural antioxidants
68 present in marjoram. A number of techniques are available for the extraction of natural
69 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 ($\cong 25^\circ\text{C}$). 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 polytetrafluoroethylene

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 Singleton 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 Na₂CO₃ (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 FeCl₃.6H₂O 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 , $^\circ\text{C}$) 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 $^\circ\text{C}$), 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
$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \dots\dots\dots(\text{Eq. 1})$$

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

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 $^{\circ}\text{C}$ (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 $^{\circ}\text{C}$ (temperature) and 11.18 to 14.89 min (time). The
290 antioxidant polyphenols namely rosmarinic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-
291 glucoside, 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 $^{\circ}\text{C}$ respectively. In all the herbs examined, the
294 optimal extraction condition for all the parameters combined was 61 μm , 35 $^{\circ}\text{C}$ 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

314 **ACKNOWLEDGEMENT**

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

316

317 **LITERATURE CITED**

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389 **This work was supported by the Irish Department of Agriculture Fisheries and**
390 **Food funded Food Institutional Research Measure and ABBEST scholarship**
391 **programme of Dublin Institute of Technology, Dublin, Ireland.**

392

393 **Figure captions**

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

397

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

401

402 Figure 3. HPLC chromatograms of UAE extracts of marjoram obtained at 61 μm , 35 $^{\circ}\text{C}$
403 and 10 min (A), in comparison to solid/liquid extracts (B) showing the changes in peaks
404 of different polyphenols (1=caffeic acid, 2=luteolin-7-O-glucoside, 3=apigenin-7-O-
405 glucoside, 4=rosmarinic acid, 5=carnosol and 6=carnosic acid).

406

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 μ m, 35 °C and 15 min	9.90	0.984	9.51 \pm 0.10	1.34	4.85 \pm 0.05
FRAP (g Trolox/100 g DW)		18.56		18.96 \pm 0.19	0.70	9.00 \pm 0.17
Rosmarinic acid (mg/g DW)		24.53		24.86 \pm 0.45	0.45	12.08 \pm 0.03
Luteolin-7- <i>O</i> -glucoside (mg/g DW)		5.38		5.27 \pm 0.14	0.65	2.69 \pm 0.02
Apigenin-7- <i>O</i> -glucoside (mg/g DW)		1.54		1.60 \pm 0.13	1.31	0.86 \pm 0.01
Caffeic acid (mg/g DW)		0.15		0.14 \pm 0.01	1.55	0.10 \pm 0.01
Carnosic acid (mg/g DW)		10.25		10.63 \pm 0.26	1.20	3.56 \pm 0.15
Carnosol (mg/g DW)		1.72		1.81 \pm 0.16	1.36	0.72 \pm 0.02

^a Data are expressed as means \pm 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- <i>O</i> -glucoside	Apigenin-7- <i>O</i> -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}	$+6.99 \times 10^{-2d}$	$+1.08 \times 10^{-2d}$	$+8.04 \times 10^{-3b}$	-1.73×10^{-3ns}	$+1.1 \times 10^{-1d}$	$+7.45 \times 10^{-3c}$
β_2 (Temperature)	-3.29×10^{-1b}	-4.49×10^{-1d}	-6.27×10^{-2d}	-1.10×10^{-1a}	$+1.23 \times 10^{-2b}$	-1.05×10^{-2ns}	-8.6×10^{-2ns}	$+5.44 \times 10^{-3ns}$
β_3 (Time)	-9.93×10^{-2a}	-5.17×10^{-1c}	$+1.9 \times 10^{-1c}$	-9.67×10^{-3ns}	$+1.20 \times 10^{-2ns}$	-1.45×10^{-3ns}	-9.93×10^{-3ns}	$+6.84 \times 10^{-3ns}$
Quadratic								
β_{11}	$+1.13 \times 10^{-4ns}$	$+3.12 \times 10^{-4ns}$	-3.97×10^{-4ns}	-2.75×10^{-4a}	-	$+1.20 \times 10^{-5ns}$	-1.27×10^{-3d}	-
β_{22}	$+4.83 \times 10^{-3b}$	$+7.39 \times 10^{-3c}$	$+3.04 \times 10^{-3b}$	$+1.22 \times 10^{-3b}$	-	$+1.50 \times 10^{-4b}$	$+9.11 \times 10^{-4a}$	-
β_{33}	-7.99×10^{-4ns}	$+2.38 \times 10^{-3ns}$	$+8.27 \times 10^{-4ns}$	-1.27×10^{-3ns}	-	-7.20×10^{-5ns}	$+2.09 \times 10^{-4ns}$	-
Cross product								
β_{12}	$+1.55 \times 10^{-3a}$	$+5.46 \times 10^{-4ns}$	$+2.27 \times 10^{-4ns}$	$+1.13 \times 10^{-3c}$	-	$+3.72 \times 10^{-5a}$	$+1.1 \times 10^{-3b}$	-
β_{13}	$+3.25 \times 10^{-4ns}$	$+5.95 \times 10^{-3b}$	-3.54×10^{-4ns}	$+5.91 \times 10^{-4ns}$	-	$+6.63 \times 10^{-5ns}$	$+1.8 \times 10^{-4ns}$	-
β_{23}	$+5.79 \times 10^{-3a}$	$+1.19 \times 10^{-2c}$	-4.61×10^{-4a}	$+6.92 \times 10^{-4ns}$	-	$+1.19 \times 10^{-4ns}$	-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 $p \leq 0.05$

^b significant at $p \leq 0.01$

^c significant at $p \leq 0.001$

^d significant at $p \leq 0.0001$

Figure 1

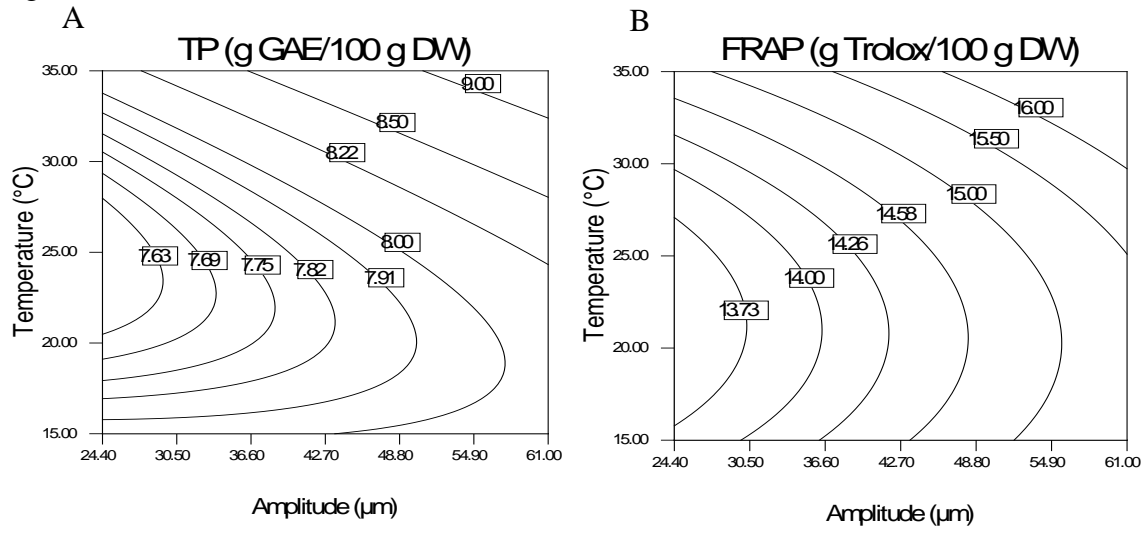


Figure 2

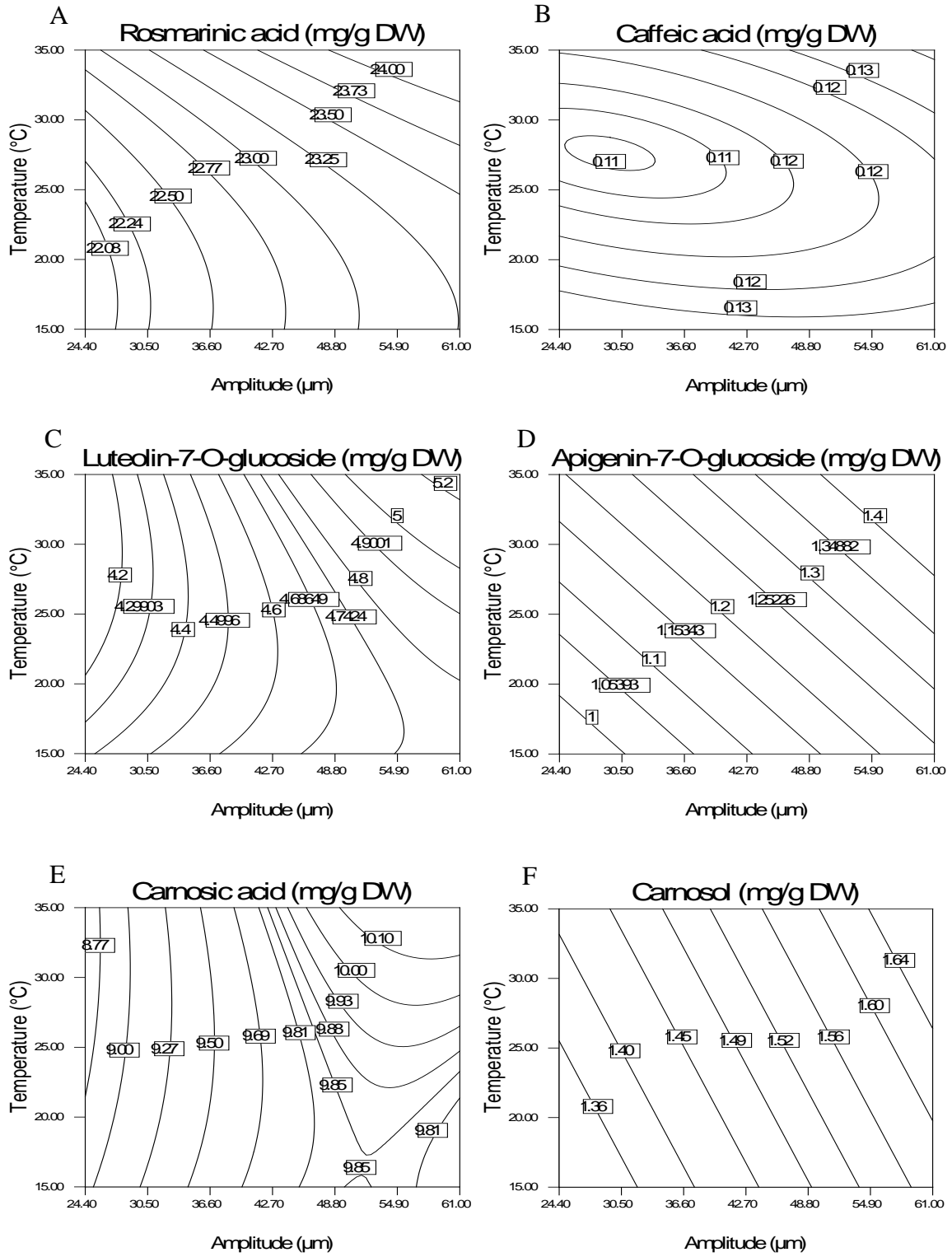


Figure 3

