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## **Optimization of conventional and ultrasound assisted extraction of flavonoids from grapefruit (*Citrus paradisi* L.) solid wastes.**

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40 **Abstract**

41 Flavonoid compounds from grapefruit wastes were obtained by conventional solid-  
42 liquid extraction (CE) and ultrasound assisted extraction (USE). Naringin was by far the  
43 most abundant flavonoid in the extracts ranging from 18-28 mg/g dw for CE and 24-36  
44 mg/g dw for USE. Response surface methodology allowed obtaining predictive models  
45 for total phenolic content (TPC) and total antioxidant activity (TAA) as a function of  
46 the process variables ethanol concentration (EtC) (defined as weight of ethanol/weight  
47 of solution), temperature (T) and time (t) with reasonable success (CE-TPC,  $R^2=0.86$ ,  
48 CE-TAA,  $R^2=0.85$ ; USE-TPC,  $R^2=0.82$ ; USE, TAA,  $R^2=0.86$ ). USE was very effective  
49 when compared with conventional solvent extraction, allowing higher extraction yields  
50 (on average TPC 50% and TAA 66% higher) with lower temperatures and extraction  
51 times. Although the optimum process conditions indicate the use of a low ethanol  
52 concentration and ultrasounds ( $T=25^{\circ}\text{C}$ ,  $\text{EtC}=0.4$  (g/g) (40 g/100 g) and  $t=55$  min  
53 leading to  $\text{TPC}=80.0$  mg GAE/g dw and  $\text{TAA}=38.3$  mmol trolox/g dw), it has been  
54 proved that an USE treatment free of organic solvent ( $\text{EtC}=0$  g/g), at moderate  
55 temperature ( $25^{\circ}\text{C}$ ) and short time ( $t=3$  min) leads to similar results ( $\text{TPC}=75.3$  mg  
56 GAE/g dw and  $\text{TAA}=31.9$  mmol trolox/g dw), suggesting its use for economic and  
57 environmental purposes.

58

59

60 **Keywords**

61 Antioxidant activity, response surface methodology, biorefinery, polyphenols, fruits.

62

63

64 **1. Introduction**

65 The vegetable and fruit industry produces large volume of solid and liquid wastes  
66 obtained from the transformation of raw materials into final products. The disposal of  
67 these wastes presents serious environmental problems due to their content on organic  
68 substances; indeed, they can be easily fermented by microorganisms producing  
69 leachates characterized by high values of chemical and biological organic demands that  
70 might access to underground-water, causing among others, eutrophization problems  
71 (Monier et al, 2010). In addition, the waste disposal does not account the potentiality of  
72 recycling value-compounds present in the food waste, what is in disagreement with the  
73 new tendencies of environment protection around the world (Kosseva, 2013).  
74 Interesting perspectives originate from the huge amount of food materials discharged  
75 worldwide and the existing technologies able to recover and recycle high-added value  
76 compounds inside the food chain (Galanakis, 2012). But according to the biorefinery  
77 concept, biomass needs a first transformation with a huge separation or extraction of  
78 plant components for a later functionalization or formulation for their use in numerous  
79 economic sectors (Octave & Thomas, 2009).

80 Grapefruit juice has long been recognized for containing bioactive compounds  
81 such as ascorbic acid, limonoids, flavonoids, carotenoids and pectin important for  
82 human health (Girenavar, Jayaprakasha & Patil, 2008). Naringin, neohesperidin,  
83 eriocitrin, hesperidin, neoeriocitrin are the major flavonoids present in the grapefruit  
84 juice (Zhang, Duan, Zang, Huang & Liu, 2011). Extensive studies have confirmed that  
85 these compounds exhibit antioxidant, anti-inflammatory, antiproliferative,  
86 anticarcinogenic and antimicrobial properties (Patil, Jayaprakasha, Chidambara &  
87 Vikram, 2009).

88           In food industries grapefruit is mainly used to make fresh juice or citrus-based  
89 drinks. The peels are often used to feed animals or are thrown away directly. In average,  
90 during the production of grapefruit juice, around the half of the grapefruit weight is  
91 separated as solid waste. Like the juice, grapefruit peels contain high amounts of  
92 antioxidant compounds (Guo, Yang, Wei, Li, Xu & Jiang, 2003) which can find  
93 application as ingredients for dietary supplements and/or as raw materials in cosmetic,  
94 pharmaceutical and nutraceutical applications.

95           Extraction represents the first step to get bioactive materials from a plant and  
96 several methods can be used to obtain these compounds from peel wastes. They include  
97 conventional solvent extraction, alkaline extraction (Bocco, Cuvelier, Richard & Berset,  
98 1998), microwave assisted extraction (Wang, Shang, Wang & Feng, 2011), resin-based  
99 extraction (Di Mauro, Fallico, Passerini & Maccarone, 2000), enzyme-assisted  
100 extraction (Li, Smith & Hossain, 2006a), subcritical water extraction (Ko, Cheigh &  
101 Chung, 2014) and supercritical fluid extraction (Giannuzzo, Boggetti, Nazareno &  
102 Mishima, 2003).

103           Most of these methods are limited by some drawbacks, such as the degradation  
104 of the compounds of interest due to high temperatures, long extraction times (as in  
105 solvent extractions) and health-related risks.

106           Ultrasound assisted-extraction is an emerging extraction technology considered  
107 as a potential alternative to traditional methods for the extraction of metabolites from  
108 plants (Galanakis, 2012). Typical advantages for the food industry in terms of  
109 economical and practical aspects include short extraction times, decrease of solvent  
110 consumption, overall enhancement of extraction rate, enhancement of the quality  
111 extracts, improvement of aqueous extraction processes where solvents cannot be used

112 and improved extraction of heat sensitive compounds which would otherwise have low  
113 yields (Vilkhu, Mawon, Simons & Bates, 2008).

114 In this work, conventional and ultrasound assisted extraction of flavonoids from  
115 grapefruit peels have been performed and compared. The effect of operating variables  
116 such as ethanol/water ratio, extraction temperature and extraction time on the yield of  
117 phenolic compounds and antioxidant activity was evaluated according to the response  
118 surface methodology approach. Optimization of multiple responses permitted to  
119 establish operating conditions giving maximum yields of phenolic compounds and the  
120 predicted results were experimentally validated.

121

## 122 **2. Materials and Methods**

### 123 *2.1. Grapefruit solid waste*

124 Grapefruits were purchased in a local market. The diameter of fruits ranged  
125 between 93-139 mm. All fruits were carefully washed and dried. Five fruits were  
126 selected randomly for moisture analysis while other grapefruits were prepared for  
127 polyphenol extraction as follows: each fruit was cut into its halves that were squeezed  
128 (HAR 2737, Philips Ibérica, Madrid) obtaining two fractions, juice and solid wastes  
129 (pulp, albedo and flavedo). These solid wastes were cut into smaller pieces of a similar  
130 size by hand and dried in an oven (Conterm, JP Selecta, Barcelona) at 60°C until  
131 constant weight. Dried solid wastes were ground in a household mill (GVX 242,  
132 KRUPS GmbH, Solingen) and sieved to obtain particles with a size lower than 1 mm.  
133 Those particles higher than 1 mm, were ground and sieved again. Dried and milled  
134 samples (moisture content=0.11 g water/g dw) were stored in hermetic containers at  
135 4±1°C until further processing.

136

137 2.2. *Flavonoids extraction.*

138 2.2.1. Experimental design

139 Flavonoids extraction was carried out under two different extraction systems:  
140 conventional solid-liquid extraction (CE) and ultrasound assisted solid-liquid extraction  
141 (USE) in Erlenmeyer flasks. For each run 5 g of dried and milled samples were used.  
142 The same solid-liquid ratio (1:8 (g:g)) was used in both treatments. Operative variables  
143 considered were: ethanol concentration (EtC) (defined as weight of ethanol/weight of  
144 solution), temperature (T) and extraction time (t).

145 Response surface methodology was used to establish the best extracting  
146 conditions (EtC, T and t) for the CE and USE treatments. Response surface  
147 methodology is less expensive and time-consuming than classical methods since several  
148 variables are simultaneously tested with a minimum number of trials in such a way that  
149 it is possible to find interactions between variables (Montgomery, 2001) and offers a  
150 large amount of information from a reduced number of experiments (Baç & Boyaci,  
151 2007).

152 A central composite design was used for each extraction system, where the  
153 dependent variables were, total polyphenol content (TPC), neoeriocitrin, narirutin,  
154 tangeritin, naringin, hesperidin and neohesperidin content and total antioxidant activity  
155 (TAA). The experiments were performed in random order. A total of 19 experiments  
156 were carried out in each central composite design: eight factorial points, six star points  
157 and five center points.

158

159 2.2.2. Conventional solid-liquid extraction (CE)

160 The experimental ranges (-1;+1) considered were: EtC, 0.2-0.8 g/g (20-80 g/100  
161 g), temperature, 34-61°C and time, 130-413 min. Coded and actual variables for each

162 experimental run are listed in Table 1. Mixtures were heated and stirred at 300 rpm  
163 during the extraction runs (magnetic stirrer LBX H20SQC, Labbox, Barcelona).

164

### 165 2.2.3. Ultrasound assisted solid-liquid extraction (USE)

166 An ultrasound bath with temperature control and working at  $40\pm 2$  kHz of  
167 frequency (ATM40-3LCD, Labbox, Barcelona) was used for the USE experiments. This  
168 system includes 2 piezo-electric steel-aluminium transducers located at the bottom of  
169 the ultrasound bath, 50 W each, adding up to 100 W of ultrasound power. A paddle  
170 stirrer (RW11, IKA Works, Staufen) was used for stirring at 300 rpm. The ranges for  
171 EtC and temperature were the same as those used in the CE experiments. However, in  
172 order to establish the range of time, preliminary experiments were done (EtC 0.5 g/g (50  
173 g/100 g); 48°C) in which the evolution of TPC in the purified extracts was followed  
174 with time course until 6 h of extraction. It was observed a hyperbolic trend reaching the  
175 asymptotic value of about 75 mg gallic acid equivalents/g dw of grapefruit at around 60  
176 min. Thus, the selected extraction time was in the range 15-48 min (Table 1).

177

### 178 2.3. Purification of crude extracts

179 Extracts obtained in both CE and USE experiments were purified as follows:  
180 after extraction, samples were transferred to centrifuge tubes and centrifuged  
181 (Medifriger BL-S, JP Selecta, Barcelona) at 500 g force for 10 min at room temperature.  
182 Supernatants were collected and vacuum filtered using filter paper Whatman 40.  
183 Afterwards, crude extracts were purified by using C18 chromatography cartridges (Sep-  
184 Pack, Waters, Milford) to remove sugars and organic acids that could interfere in the  
185 total polyphenol content analysis (Li, Smith & Hossain, 2006b). Purified extracts were  
186 stored at -20°C until their analysis.



187

## 188 2.4. Analytical determinations

### 189 2.4.1. Moisture

190 Five randomly selected grapefruits were cut and squeezed as described in 2.1.  
191 Solid wastes were cut by hand in pieces of similar size and then were weighted.  
192 Afterwards, samples were introduced in a vacuum oven (Vacioterm, JP Selecta,  
193 Barcelona) at 60°C and less than 13 kPa until constant weight. Moisture content was  
194 determined from the weight difference before and after dehydration.

195

### 196 2.4.2. Total polyphenols content (TPC)

197 The TPC analysis was carried out using the Folin-Ciocalteu method (Pinelo,  
198 Rubilar, Sineiro & Núñez, 2004). This method consisted in the addition of 0.5 mL of the  
199 purified extract with 2 mL of an aqueous solution of sodium carbonate (7.5 g/100 mL)  
200 and 2.5 mL of Folin-Ciocalteu reactant previously diluted 10 folds. The mixture was  
201 stirred in a vortex and left stand for 15 min at room temperature. Afterwards the  
202 absorbance was measured at 765 nm. A calibration curve was done using gallic acid as  
203 standard, and results were expressed in terms of gallic acid equivalents/mL (mg  
204 GAE/mL) and recalculated as mg GAE/g sample dw.

205

### 206 2.4.3. HPLC analysis

207 Flavonoid content was determined by a HPLC system (Waters Alliance 2695,  
208 Milford), equipped with a vacuum degasser, a binary pump, an autosampler, a  
209 thermostated column compartment, a model 2996 diode array detector and a Empower  
210 software for data collection from Waters Corporation. The chromatographic separation

211 was performed by using a Luna C 18(2) column (250×4.6 mm, 5 μm, Phenomenex,  
212 Torrance).

213 The mobile phase (v/v) consisted of 1 mL/L of formic acid in water (eluent A)  
214 and 1 mL/L of formic acid in acetonitrile (eluent B). The following gradient (v/v)  
215 system was used: 0 min, 90% A and 10% B; 30 min, 50% A and 50% B; 35 min, 0% A  
216 and 100% B. Analyses were stopped after 50 min. The system was equilibrated between  
217 runs for 10 min using the start mobile phase composition. The flow was maintained at 1  
218 mL/min and the injection volume was 0.01 mL. Diode array detection was between 200  
219 and 600 nm, and absorbance was recorded at 280 nm.

220 Prior to HPLC analysis all samples were filtered by using 0.45 μm nylon filters.  
221 Flavonoids were identified by matching the retention time and their spectral  
222 characteristics against those of standards. Quantification was made according to the  
223 linear calibration curves of standard compounds: neoriocitrin, narirutin, naringin,  
224 hesperidin and neohesperidin and recalculated as mg/g sample dw. HPLC  
225 measurements were performed as one replicate.

226

#### 227 2.4.4. Total antioxidant activity (TAA)

228 The total antioxidant activity (TAA) of the purified extracts was analyzed  
229 according to the DPPH\* (2,2-diphenyl-1-picrylhydrazyl radical) method (Arnous,  
230 Makris, & Kefalas, 2002; Roussis, Lambropoulos, Tzimas, Gkoulioti, Marinos,  
231 Tsoupeis et al., 2008). The ability of samples to scavenge the DPPH\* was evaluated as  
232 follows: 0.2 mL of the extract and 3.8 mL of a solution of DPPH\* in methanol 0.06  
233 mmol/L were added to test tubes; the antioxidant activity was determined as the  
234 inhibition percentage of the DPPH\* due to the antioxidant compounds present in the

235 sample measuring the absorbance at 515 nm from t=0 min to t=30 min of reaction. The  
236 inhibition percentage was calculated according to the following equation:

$$237 \quad \% \Delta Abs_{515} = \frac{Abs_{515(0)} - Abs_{515(30)}}{Abs_{515(0)}} \cdot 100 \quad (1)$$

238 A previous calibration curve was done using Trolox (6-hydroxy-2,5,7,8-  
239 tetramethylchroman-2-carboxylic acid) solutions in a range 0-0.5 mmol/L. Thus, results  
240 of antioxidant activity were expressed as mmol/L equivalents of trolox and recalculated  
241 as mmol trolox/g sample dw.

242

### 243 2.5. Statistical analysis

244 All the TPC and TAA analyses were done in triplicate and results were  
245 expressed as the mean value of three measurements. Experimental data of each central  
246 composite design were submitted to response surface analysis using the software  
247 “Statgraphics” (version Centurion XVI, StatPoint Technologies, Inc.). Linear, quadratic  
248 and interaction effects of the three variables considered (EtC, t and T) on the response  
249 variables were calculated. Their significance was evaluated by analysis of variance  
250 (ANOVA). Each response surface was obtained by fitting experimental data to a  
251 second-order polynomial model according to equation (2):

$$252 \quad Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{j=i+1}^n \beta_{ij} X_i X_j \quad (2)$$

253 where  $Y$  is the studied response and  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  are the independent, linear,  
254 quadratic and interaction coefficients, respectively. Coefficients of determination ( $R^2$ )  
255 were obtained for each fit.

256 Multiple response optimization was performed through the use of the desirability  
257 function (D) (Montgomery, 2001) by using the Statgraphics software, to find the  
258 extraction conditions leading to compromise levels of TPC and TAA. Desirability was  
259 obtained through Eq. (3):

$$D = (d_1(Y_1) \cdot d_2(Y_2))^{1/2} \quad (3)$$

Where  $d_i(Y_i)$  are the normalized values (from 0 to 1) of each of the studied responses (TPC  $i=1$ ; TAA  $i=2$ ). Then  $D=0$  corresponds to the lowest desirability and  $D=1$  to the highest desirability.

264

### 265 3. Results and discussion

#### 266 3.1 Conventional solid-liquid extraction.

267 In Table 2 are listed the results of the conventional extraction in terms of total  
268 polyphenol content, total antioxidant activity and some selected flavonoid compounds.  
269 TPC ranged between 25.3-55.8 mg GAE/g dw. The mean TPC value for the 5 center  
270 points (EtC=0.5 g/g (50 g/100 g), T=48°C, t=270 min (4.5 h); runs 2, 5, 9, 14 and 16)  
271 was 36.5±4.2 mg GAE/g dw. The minimum value was obtained in run 7 (EtC=0.8 g/g  
272 (80 g/100 g), T=34°C, t=413 min (6.9 h)), while the maximum TPC was obtained in run  
273 8 (EtC=0.2 g/g (20 g/100 g), T=34°C, t=413 min (6.9 h)). Experimental conditions in  
274 these two experiments differed only in the EtC, so that low EtC seems to have a positive  
275 effect on total polyphenol extraction. On the other hand, total antioxidant activity  
276 ranged between 4.0 mmol trolox/g dw in run 11 (EtC=1.0 g/g (100 g/100 g), T=48°C,  
277 t=270 min (4.5 h)) and 23.0 mmol trolox/g dw in run 2 (EtC=0.5 g/g, T=48°C, t=270  
278 min (4.5 h)), and the mean TAA for the center points was 18.8±4.0 mmol trolox/g dw.

279 Naringin (18-28 mg/g dw) was by far the most abundant flavonoid  
280 independently of the experimental conditions used. Hesperidin (0.23-0.74 mg/g dw) and  
281 narirutin (0.28-0.70 mg/g dw) were the following most abundant flavonoids. These  
282 results are in agreement with those reported by other authors on the main flavonoids in  
283 grapefruit (Xu, Ye, Chen & Liu, 2007; Zhang et al., 2011; Goulas & Mangaris, 2012)  
284 where naringin ranged (14.194-35.240 mg/g dw), hesperidin (1.635-3.869 mg/g dw) and

285 narirutin (0.845-5.216 mg/g dw). Differences may be due to the fruit variety and  
286 method of extraction. The other polyphenols were present in less quantity, being their  
287 order naringin>>hesperidin=narirutin > neohesperidin=neohesperidin > tangeritin.

288         The main factors affecting the phenolic content of the extract (TPC) (Table 3)  
289 are the EtC (negative effect,  $p=0.0003$ ) and temperature (positive effect,  $p=0.0127$ )  
290 (Pareto plot (Figure 1(a)); in other words, the increase of temperature enhances the  
291 extraction of flavonoids but high ethanol concentration does not favour the extraction.  
292 Several authors obtained the same trend in terms of ethanol concentration: the presence  
293 of ethanol in the solvent has a positive effect on the polyphenol extraction until a  
294 maximum ethanol concentration, from this point the polyphenol extraction decreases  
295 (Turkmen, Sari & Velioglu, 2006; Yang, Jiang, Li, Chen, Wang & Zhu, 2009;  
296 Rodríguez Amado, Franco, Sánchez, Zapata & Vázquez, 2014). Ethanol reduces the  
297 dielectric constant of the solvent, thus enhancing the solubility and diffusion of  
298 polyphenols. However, highly pure organic solvents, e.g. 100% ethanol, may lead to  
299 dehydration and collapse of the vegetable cells and denaturation of cell wall proteins,  
300 making difficult the diffusion of polyphenols from the plant material to the extracting  
301 liquid (d'Alessandro, Kriiaa, Nikov & Dimitrov, 2012; Amendola, De Faveri & Spigno,  
302 2010; Librán, Mayor, Garcia-Castello & Vidal-Brotons, 2013). This effect can be  
303 observed in the surface response of TPC as a function of temperature and EtC at a  
304 constant time of 190 min (Figure 1(b)), where it is also observed that the negative effect  
305 of the EtC is more accentuated at low temperatures. Figure 1(a) and Table 3 also show  
306 that the main variables affecting the antioxidant activity of the extracts (TAA) are the  
307 EtC (negative,  $p=0.0006$ ) and the quadratic effect of EtC (negative,  $p= 0.0031$ ). The  
308 effect of these variables can be observed in the surface response of Figure 1(c);

309 temperature has not a clear effect, but TAA dramatically decreases with high values of  
310 EtC.

311 Regression coefficients of the response surface equations are listed in Table 3.  
312 For both TPC and TAA the fits were satisfactory ( $R^2=0.86$  and  $R^2=0.85$  respectively).  
313 Multiresponse optimization was performed so as to obtain the best process conditions  
314 for a compromise between the best TPC and TAA values. A maximum desirability  
315  $D=1.0$  was obtained for the process conditions:  $t=190$  min;  $T=69$  °C and  $EtC=0.3$  g/g,  
316 being the predicted responses  $TPC=56.0$  mg GAE/g dw and  $TAA=23.5$  mmol trolox/g  
317 dw. Figure 1(d) represents the contour plot of the D values as a function of temperature  
318 and EtC, for a constant time of 190 min. As observed, the highest D values are obtained  
319 for low EtC and high temperatures.

320

### 321 *3.2 Ultrasound assisted solid-liquid extraction.*

322 In USE, total polyphenol content ranged between 29.4-80.0 mg GAE/g dw  
323 (Table 4). Run 11 ( $EtC=1$  g/g (100 g/100 g),  $T=48$ °C,  $t=32$  min) gave the lowest TPC  
324 while runs 1 and 4 ( $EtC=0.5$  g/g (50 g/100 g),  $T=70$ °C,  $t=32$  min and  $EtC=0.5$  g/g (50  
325 g/100 g),  $T=48$ °C,  $t=60$  min, respectively) showed highest values, suggesting that, as  
326 observed in conventional extraction, a high EtC has a negative effect on the TPC. Time  
327 had a positive effect but it was not significant at the time range studied because high  
328 extraction yields were found at very short times, very similar to that obtained for longer  
329 times In this sense, run 15 ( $T=48$ °C,  $EtC=0.5$  g/g (50 g/100 g),  $t=3$  min) gave a TPC  
330 value of 70.2 mg GAE/g dw whereas the mean of the center point (runs 2, 5, 9, 14 and  
331 16 ( $T=48$ °C,  $EtC=0.5$  g/g (50 g/100 g),  $T=32$  min) gave a TPC value of  $71.0\pm 8.5$  mg  
332 GAE/g (d.w). This is also corroborated by the Pareto plot (figure 2(a)). Similar behavior  
333 was found during USE of polyphenols from black chokeberry wastes (d'Alessandro,

334 Dimitrov, Vauchel & Nikov, 2014), and during USE of polyphenols from grape (Da  
335 Porto, Porretto & Decorti, 2013). It seems that the use of ultrasounds enhances  
336 dramatically the extraction rate and equilibrium is attained at short times. Regarding the  
337 temperature, when comparing run 10 (T=25°C, EtC=0.5 g/g (50 g/100 g), t=32 min;  
338 TPC=71.1 mg GAE/g dw), the mean of the center point (T=48°C, EtC=0.5 g/g (50  
339 g/100 g), t=32 min; TPC=71.0±8.5 mg GAE/g dw) and run 1 (T=70°C, EtC=0.5 g/g (50  
340 g/100 g), t=32 min; TPC=80.0 mg GAE/g dw), it is observed a positive effect although it  
341 is not significant. This can be corroborated also by the Pareto plot (figure 2a).  
342 Temperature has two opposite effects, since it enhances mass transfer during extraction  
343 but also promotes higher degradation rates.

344         The lowest TAA was found for the run 11 (EtC=1.0 g/g (100 g/100 g), T=48°C,  
345 t=32 min, 12.5 mmol trolox/g dw) in agreement with the lowest TPC. However, runs 8  
346 and 19 (EtC=0.2 g/g (20 g/100 g), T=34°C) showed approximately the same maximum  
347 TAA (28.3 mmol trolox/g dw in average), suggesting that moderate ethanol  
348 concentrations and temperatures lead to higher antioxidant activity in the extracts. The  
349 mean of the center point (runs 2, 5, 9, 14 and 16 (T=48°C, EtC=0.5 g/g (50 g/100 g),  
350 T=32 min) gave a TAA value of 26.7±3.7 mmol trolox/g dw.

351         As expected (see Table 4) naringin was, by far, the most abundant flavonoid in  
352 the ultrasound assisted extractions (24-36 mg/g dw) followed by hesperidin and  
353 narirutin, which showed similar contents (0.72-1.14 mg/g dw and 0.42-0.98 mg/g dw,  
354 respectively). The order of flavonoids by quantity is the same as that found in  
355 conventional extraction treatments.

356         ANOVA showed that linear and quadratic EtC effects were statistically  
357 significant and negative for the TPC (p=0.0340 and p=0.0005, respectively) as shown in  
358 the Pareto plot of the Figure 2(a). The same trend was observed for TAA with p-values

359 of 0.0017 and 0.0005, respectively. TPC surface response (Fig. 2(b)) at t=55 min shows  
360 that temperature had no significant effect on the phenolic content of the extract, whereas  
361 intermediate values of the EtC (0.4-0.6 g/g (40-60 g/100 g)) led to the highest TPC  
362 values. Regarding TAA, the corresponding response surface (Fig. 2(c) at t=55 min)  
363 shows that the highest antioxidant activities were found for intermediate values of the  
364 EtC, whereas temperature had only a slight negative effect at low EtC. Regression  
365 coefficients of TPC and TAA response surfaces are detailed in Table 3, where is also  
366 observed that fits of Eq. (2) to experimental data were satisfactory in both cases (TPC,  
367  $R^2=0.82$  and TAA,  $R^2=0.86$ ). Regarding multiresponse optimization, a maximum  
368 desirability  $D=1$  was obtained for the process conditions: t=55 min; T=25 °C and  
369 EtC=0.4 g/g (40 g/100 g), being the predicted responses TPC=80.0 mg GAE/g dw and  
370 TAA=38.3 mmol trolox/g dw. Figure 2(d) shows the contour plot of the D values as a  
371 function of T and EtC, for a constant time of t=55 min. As observed, the best  
372 desirability values are located at intermediate values of the ethanol concentration, being  
373 slightly higher for low temperatures.

374

375 *3.3 Comparison of conventional and ultrasound assisted extraction, selection of process*  
376 *conditions and validation.*

377 On average, TPC and TAA values in USE were about 1.7 folds the value  
378 obtained in CE in both cases, despite the shorter times of extraction in USE. This is in  
379 agreement with results obtained by several authors in the extraction of polyphenols  
380 from different fruits and vegetables (d'Alessandro et al., 2012; Da Porto et al., 2013;  
381 Khan, Abert-Vian, Fabiano-Tixier, Dangles & Chemat, 2010). The effect of the  
382 ultrasounds is attributed to their interaction with the plant material, altering its physical  
383 and chemical properties, and to the cavitation effect which facilitates the release of



384 extractable compounds and enhances the mass transport by disrupting the plant cell  
385 walls (Chemat, Huma & Khan, 2011). In both cases, the flavonoid profile was similar,  
386 with naringin as the most abundant flavonoid followed far away by hesperidin and  
387 narirutin. Neohesperidin, neohesperin and tangeretin were found in very low amounts  
388 when compared to the other flavonoids.

389 Both temperature and ethanol concentration had significant effect on TPC for  
390 conventional extraction. Only EtC (linear and quadratic terms) had significant effect on  
391 TAA for conventional extraction and on TPC and TAA for ultrasound assisted  
392 extraction. Time was found to be a not significant variable in this study; this is adequate  
393 considering the wide ranges of time tested, although shorter ranges of time should be  
394 used in later studies to verify this trend. A positive correlation between total polyphenol  
395 content and total antioxidant activity was found ( $R^2=0.79$ ) for both conventional and  
396 ultrasound assisted extractions.

397 For the selection of the most suitable alternative of processing, and considering  
398 the process conditions selected after multiresponse optimization (CE:  $T=69^\circ\text{C}$ ,  $\text{EtC}=0.3$   
399  $\text{g/g}$  (30  $\text{g}/100$   $\text{g}$ ) and  $t=190$   $\text{min}$ ; USE:  $T=25^\circ\text{C}$ ,  $\text{EtC}=0.4$   $\text{g/g}$  (40  $\text{g}/100$   $\text{g}$ ) and  $t=55$   $\text{min}$ ),  
400 it is noticeable that for the USE higher predicted extraction yields are obtained (56.0 vs.  
401 80.0  $\text{mg GAE/g dw}$ , 23.5 vs. 38.3  $\text{mmol trolox/g dw}$ , for CE and USE respectively) and  
402 the requirements in terms of temperature and time (energy requirements) are lower, but  
403 however a higher ethanol concentration is needed.

404 An interesting option from an economical and environmental point of view  
405 would be that in which the use of ethanol is avoided in an ultrasound assisted treatment.  
406 If the ethanol concentration is fixed to 0 for the USE, the new desirability function for  
407 the multivariable optimization decreases till  $D=0.892$  and the new operative conditions  
408 are  $T=25^\circ\text{C}$  and  $t=3$   $\text{min}$ , leading to expected response variables of  $\text{TPC}=69.7$   $\text{mg}$

409 GAE/g dw and TAA=35.0 mmol trolox/g dw. These values are still better than the best  
410 obtained during the CE treatments.

411 This selection was validated carrying out the extraction under the new  
412 experimental conditions (T=25°C, EtC=0 and t=3 min). Experimental results were  
413 satisfactory, being TPC slightly higher than expected (108%), and TAA slightly lower  
414 (91%). Regarding the flavonoid analysis, it was found the same trend observed in the  
415 previous CE and USE treatments: (naringin (29 mg/g dw)>>hesperidin (0.82 mg/g  
416 dw)=narirutin (0.74 mg/g dw)>neohesperidin (0.11 mg/g  
417 dw)>tangeritin (0.017 mg/g dw)).

418

419

#### 420 **4. Conclusions**

421 Ultrasound assisted extraction of flavonoids from grapefruit wastes was found to  
422 be very effective when compared with conventional solvent extraction, allowing higher  
423 extraction yields with lower temperature and extraction time.

424 Response surface methodology permitted to develop prediction models for total  
425 phenolic content and total antioxidant activity with good correlation coefficients and to  
426 assess the effect of process variables in the extraction yields; being ethanol  
427 concentration and temperature the variables affecting the process for conventional  
428 extraction and ethanol concentration for ultrasound assisted extraction. Flavonoids  
429 composition in extracts was very similar for both treatments, being naringin by far the  
430 most abundant flavonoid in the extracts.

431 Although the optimum process conditions indicate the use of a low ethanol  
432 concentration and ultrasounds, it has been proved that an ultrasound extraction free of

433 organic solvent and moderate temperature (25°C) leads to similar results, suggesting its  
434 use for economic and environmental purposes.

435

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437

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439 financial support through the project 1965 (PAID-05-11).

440

441

#### 442 **List of symbols**

443 ANOVA analysis of variance

444 CE conventional solid/liquid extraction

445 dw dried weight

446 D desirability function

447 DPPH\* 2,2-diphenil-1-picrilhydracyl radical

448 EtC ethanol concentration (ethanol weight/solution weight (g/g))

449 GAE gallic acid equivalents

450  $R^2$  coefficient of determination

451  $s_{CP}$  standard deviation of center points

452  $s_p$  pooled standard deviation

453 T temperature

454 t time

455 TAA total antioxidant activity

456 TPC total polyphenols content

457 USE ultrasound assisted solid/liquid extraction

458  $\bar{X}_{CP}$  mean of center points

459

460 Greek symbols

461  $\beta_0$  independent coefficient  
462  $\beta_i$  linear coefficient  
463  $\beta_{ii}$  quadratic coefficient  
464  $\beta_{ij}$  interaction coefficient

465

## 466 **References**

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563

564

565 Table 1. Central composite design for the conventional and ultrasound assisted solid-liquid

566 extraction of flavonoids from grapefruit peel. Coded and actual variables.

567

Run No.	Coded variables			Actual variables			
	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Ethanol concentration (g/100 g)	Temperature (°C)	Time (min)	
						CE	USE
1	0	1.682	0	50	70	270	32
2	0	0	0	50	48	270	32
3	1.0	1.0	-1.0	80	61	130	15
4	0	0	1.682	50	48	510	60
5	0	0	0	50	48	270	32
6	-1.0	1.0	1.0	20	61	413	48
7	1.0	-1.0	1.0	80	34	413	48
8	-1.0	-1.0	1.0	20	34	413	48
9	0	0	0	50	48	270	32
10	0	-1.682	0	50	25	270	32
11	1.682	0	0	100	48	270	32
12	-1.0	1.0	-1.0	20	61	130	15
13	1.0	-1.0	-1.0	80	34	130	15
14	0	0	0	50	48	270	32
15	0	0	-1.682	50	48	30	3
16	0	0	0	50	48	270	32
17	-1.682	0	0	0	48	270	32
18	1.0	1.0	1.0	80	61	413	48
19	-1.0	-1.0	-1.0	20	34	130	15

568

Table 2. Experimental results obtained in the conventional solid-liquid extraction of flavonoids experiments from grapefruit peel

Run No.	EtOH (g/100 g)	Temp. °C	Time min	TPC <sup>a,c</sup> mg GAE <sup>d</sup> /g dw	TAA <sup>b,c</sup> mmol trolox/g dw	Neohesperidin mg/g dw	Neoriterocin mg/g dw	Narirutin mg/g dw	Naringin mg/g dw	Hesperidin mg/g dw	Tangeritin mg/g dw	
1	50	70	270	54.5	21.0	0.086	0.16	0.70	24	0.74	0.010	
2	50	48	270	43.4	23.0	0.062	0.11	0.49	24	0.55	0.011	
3	80	61	130	38.3	7.8	0.053	0.09	0.59	24	0.53	0.013	
4	50	48	510	34.1	9.4	0.052	0.10	0.44	23	0.56	0.013	
5	50	48	270	31.1	20.3	0.048	0.08	0.42	19	0.42	0.011	
6	20	61	413	43.4	20.9	0.041	0.09	0.60	21	0.56	0.011	
7	80	34	413	25.3	4.5	0.044	0.07	0.28	18	0.48	0.008	
8	20	34	413	55.8	17.4	0.076	0.09	0.61	25	0.58	0.016	
9	50	48	270	37.5	16.6	0.053	0.10	0.48	21	0.53	0.010	
10	50	25	270	37.9	20.6	0.058	0.09	0.50	22	0.52	0.012	
11	100	48	270	27.9	4.0	0.027	0.03	0.29	18	0.23	0.008	
12	20	61	130	53.2	21.7	0.076	0.14	0.70	24	0.66	0.012	
13	80	34	130	26.4	5.6	0.049	0.08	0.41	22	0.47	0.013	
14	50	48	270	35.3	17.6	0.045	0.08	0.40	23	0.42	0.012	
15	50	48	30	42.6	19.0	0.059	0.11	0.52	23	0.59	0.011	
16	50	48	270	35.3	16.5	0.043	0.08	0.37	21	0.41	0.012	
17	0	48	270	50.7	13.2	0.083	0.13	0.62	28	0.64	0.011	
18	80	61	413	40.4	7.4	0.047	0.09	0.53	26	0.47	0.016	
19	20	34	130	38.3	18.0	0.041	0.09	0.43	21	0.49	0.011	
	$s_p^e$			0.8	1.9							
	$\bar{X}_{CP}^f$	50	48	270	36.5	18.8	0.050	0.09	0.43	22	0.47	0.011
	$s_{CP}^g$	50	48	270	4.2	4.0	0.008	0.01	0.05	2	0.07	0.001

570 <sup>a</sup>Total polyphenol content; <sup>b</sup>total antioxidant activity; <sup>c</sup>TPC and TAA are expressed as mean of triplicates; <sup>d</sup>gallic acid equivalents; <sup>e</sup>pooled standard deviation; <sup>f</sup>mean of center points. <sup>g</sup>standard  
571 deviation of center points

573

574

Table 3. Regression coefficients of the fitted polynomial equations (Eq. 2) for total polyphenol content and total antioxidant activity as a function of the

575

ethanol concentration, temperature and time. Conventional and ultrasound assisted extractions of flavonoids from grapefruit wastes.

576

Regression coefficients <sup>a</sup>	Total polyphenol content						Total antioxidant activity					
	Conventional extraction			Ultrasound assisted extraction			Conventional extraction			Ultrasound assisted extraction		
	Eq.(2) coefficients	F-ratio	p-value	Eq.(2) coefficients	F-ratio	p-value	Eq.(2) coefficients	F-ratio	p-value	Eq. (2) coefficients	F-ratio	p-value
Independent												
$\beta_0$	82.7453			96.9826			12.5039			39.1254		
Linear												
$\beta_1$	-0.5670	25.29	0.0003 <sup>b</sup>	0.6831	6.73	0.0340 <sup>b</sup>	0.3078	26.43	0.0006 <sup>b</sup>	0.0840	19.41	0.0017 <sup>b</sup>
$\beta_2$	-1.6211	8.46	0.0127 <sup>b</sup>	-1.3107	0.01	0.9316	-0.1219	0.98	0.3492	-0.1860	0.89	0.3701
$\beta_3$	0.0484	0.08	0.7860	-0.5801	0.17	0.6904	0.0397	2.18	0.1741	0.0745	0.24	0.6352
Quadratic												
$\beta_{11}$	-0.0011	0.22	0.6517	-0.0107	29.15	0.0005 <sup>b</sup>	-0.0044	16.08	0.0031 <sup>b</sup>	-0.0039	27.55	0.0005 <sup>b</sup>
$\beta_{22}$	0.0179	4.71	0.0581	0.0113	1.33	0.2791	0.0023	0.18	0.6797	0.0009	0.06	0.8160
$\beta_{33}$	0.00002	0.05	0.8222	0.0065	1.10	0.3223	-0.4001	3.88	0.0805	0.0003	0.01	0.9057
Crossproduct												
$\beta_{12}$	0.0106	2.66	0.1375	0.0036	0.36	0.5632	-0.0007	0.05	0.8303	0.0035	2.61	0.1403
$\beta_{13}$	-0.0005	0.19	0.6706	0.0022	0.24	0.6351	-0.00001	0.00	0.9847	0.0014	0.67	0.4331
$\beta_{23}$	-0.0009	2.57	0.1437	0.0021	0.03	0.8736	0.00003	0.00	0.9618	-0.0038	1.01	0.3408
Regression												
$R^2$ (%)	85.8			82.6			84.5			85.7		
$R^2$ adj.(%)	66.1			65.5			69.0			71.5		

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<sup>a</sup>where 0 = intercept; 1 = Ethanol concentration (g/100 g). 2 = temperature (°C), 3 = time (min), <sup>b</sup>significant at  $\alpha=0.05$ 

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Table 4. Experimental results obtained in the ultrasound assisted extraction of flavonoids experiments from grapefruit peel.

Run No.	EtOH conc. (g/100 g)	Temp °C	Time min	TPC <sup>a,c</sup> mg <sup>d</sup> GAE/g dw	TAA <sup>b,c</sup> mmol trolox/g dw	Neohesperidin mg/g dw	Neeritrocin mg/g dw	Narirutin mg/g dw	Naringin mg/g dw	Hesperidin mg/g dw	Tangeritin mg/g dw	
1	50	70	32	80.0	26.6	0.15	0.18	0.95	27	1.14	0.013	
2	50	48	32	74.5	26.5	0.11	0.16	0.86	26	0.94	0.022	
3	80	61	15	61.7	23.6	0.09	0.12	0.87	24	0.73	0.012	
4	50	48	60	80.0	26.6	0.15	0.18	0.98	33	1.00	0.014	
5	50	48	32	69.0	26.3	0.12	0.16	0.87	32	0.95	0.012	
6	20	61	48	61.7	21.7	0.09	0.16	0.92	31	0.87	0.012	
7	80	34	48	62.6	23.2	0.09	0.17	0.90	32	0.87	0.013	
8	20	34	48	70.2	28.2	0.11	0.18	0.72	31	0.83	0.015	
9	50	48	32	68.5	26.5	0.11	0.16	0.84	26	0.91	0.014	
10	50	25	32	71.1	25.8	0.12	0.15	0.80	36	0.88	0.012	
11	100	48	32	29.4	12.5	0.04	0.05	0.42	28	0.86	0.020	
12	20	61	15	68.1	26.5	0.16	0.15	0.73	27	0.87	0.013	
13	80	34	15	66.4	23.1	0.10	0.17	0.93	27	0.99	0.009	
14	50	48	32	64.7	26.0	0.11	0.15	0.79	28	0.87	0.015	
15	50	48	3	70.2	25.5	0.10	0.18	0.87	28	0.88	0.011	
16	50	48	32	78.3	28.3	0.10	0.17	0.77	30	0.94	0.012	
17	0	48	32	56.6	24.0	0.11	0.16	0.85	30	0.85	0.011	
18	80	61	48	66.4	23.0	0.10	0.17	0.98	33	1.00	0.013	
19	20	34	15	71.9	28.3	0.09	0.08	0.72	25	0.72	0.014	
	$s_p^e$			3.9	2.7							
	$\bar{X}_{CP}^f$	50	48	32	71.0	26.7	0.11	0.16	0.83	28	0.93	0.015
	$s_{CP}^g$	50	48	32	8.5	3.7	0.01	0.01	0.04	3	0.03	0.004

581 <sup>a</sup>Total polyphenol content; <sup>b</sup>total antioxidant activity; <sup>c</sup>TPC and TAA are expressed as mean of triplicates; <sup>d</sup>gallic acid equivalents; <sup>e</sup>pooled standard deviation; <sup>f</sup>mean of center points. <sup>g</sup>standard  
582 deviation of center points

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585

586 Figure 1.

587 Conventional solid-liquid extraction of flavonoids from grapefruit peels: (a) Standardized

588 Pareto chart for total polyphenol content (TPC) and total antioxidant activity (TAA); (b)

589 TPC response surface at  $t=190$  min. where dots represent experimental data; (c) TAA

590 response surface at  $t=190$  min; (d) Desirability function chart at  $t=190$  min, where the

591 black point indicates the coordinates of the optimized conditions ( $E_tC=30$  g/100 g (0.3

592 g/g);  $T=69^\circ\text{C}$  ;  $t=190$  min).

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596 Figure 2.

597 Ultrasound assisted solid-liquid extraction of flavonoids from grapefruit peels: (a)

598 Standardized Pareto chart for total polyphenol content (TPC) and total antioxidant

599 activity (TAA); (b) TPC response surface at  $t=55$  min. where dots represent

600 experimental data; (c) TAA response surface at  $t=55$  min; (d) Desirability function chart

601 at  $t=55$  min, where the black point indicates the coordinates of the optimized conditions

602 ( $E_tC=40$  g/100 g (0.4 g/g);  $T=25^\circ\text{C}$  ;  $t=55$  min).

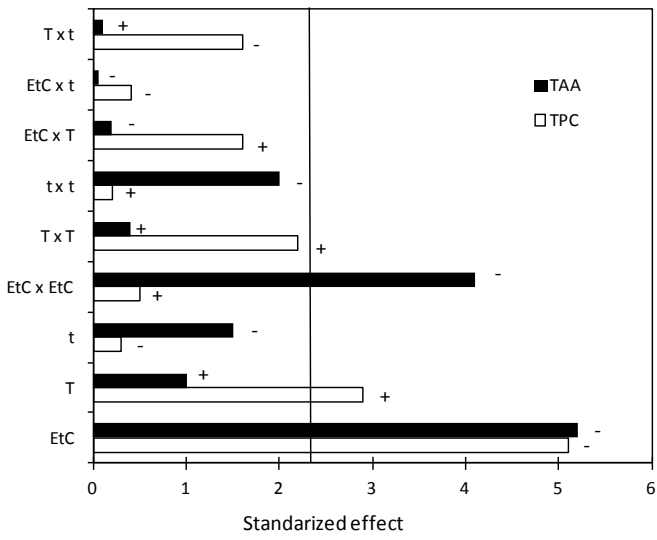
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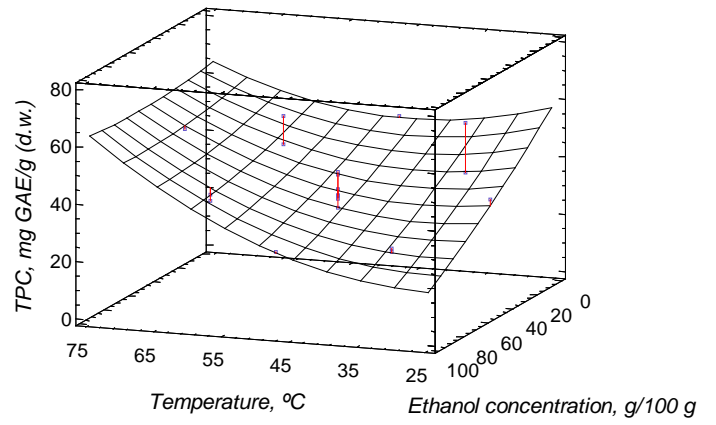
605

606 FIGURE 1

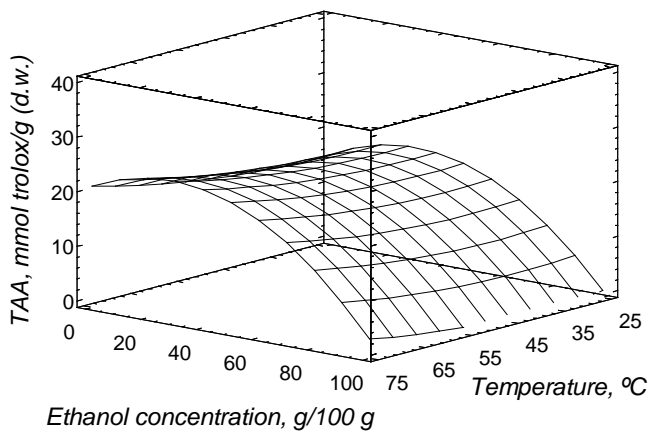
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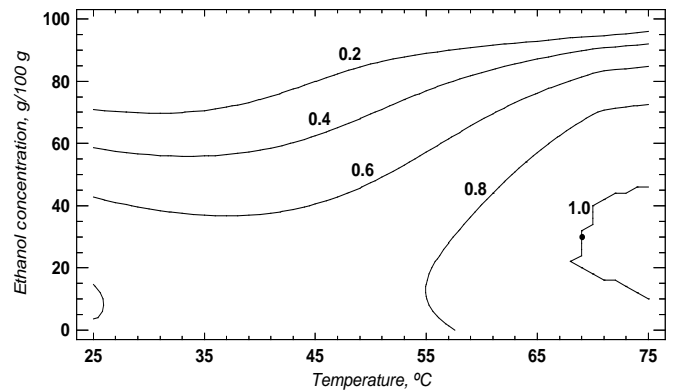
(a)



(b)



(c)



(d)

608

609

610

611

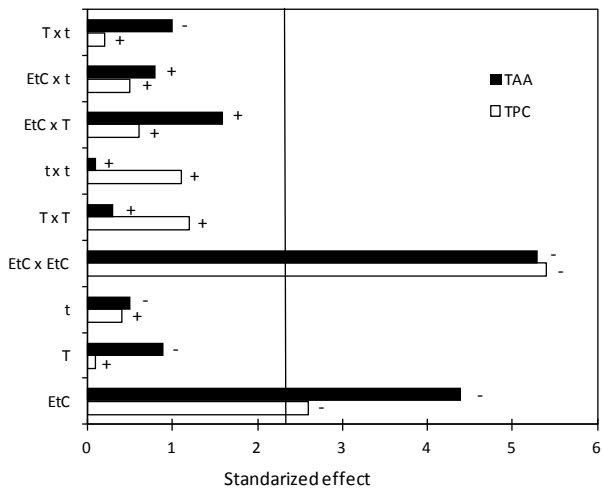
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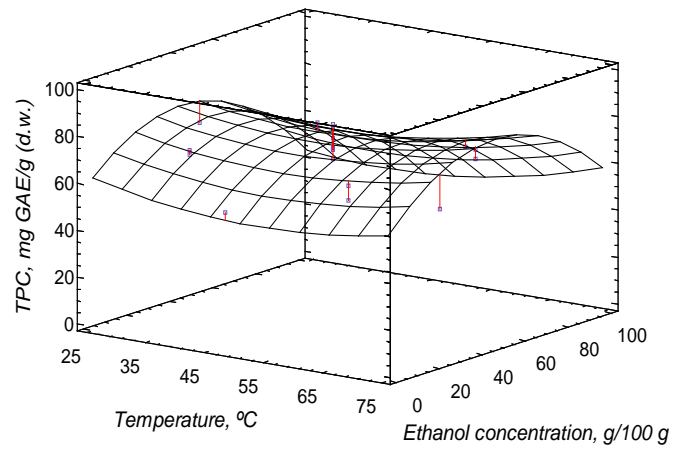
614 FIGURE 2

615

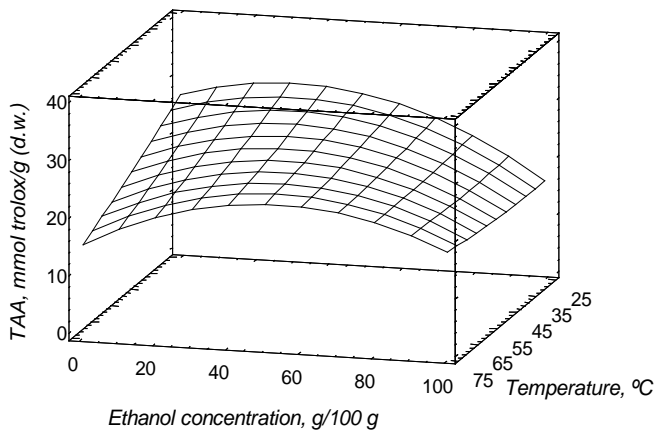
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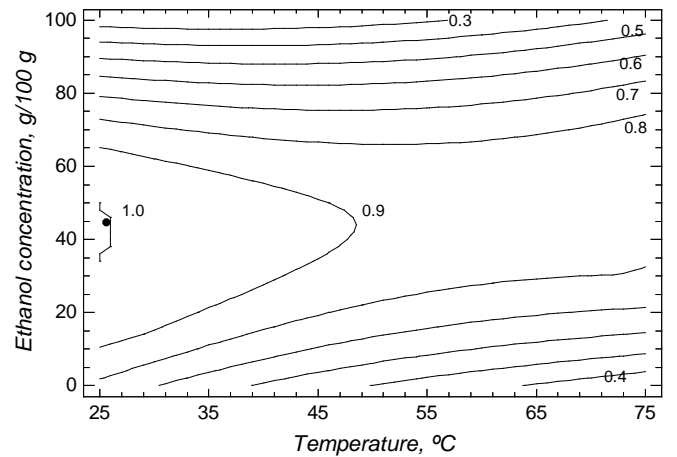
(a)



(b)



(c)



(d)

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