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

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Abstract

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

Keywords

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

1. Introduction

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

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

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

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

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

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

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

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

2. Materials and Methods

2.1. Grapefruit solid waste

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

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, 34-61ºC and time, 130-413 min. Coded and actual variables for each

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

2.2.3. Ultrasound assisted solid-liquid extraction (USE)

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

2.3. Purification of crude extracts

Extracts obtained in both CE and USE experiments were purified as follows: after extraction, samples were transferred to centrifuge tubes and centrifuged (Medifriger BL-S, JP Selecta, Barcelona) at 500 g force for 10 min at room temperature. Supernatants were collected and vacuum filtered using filter paper Whatman 40. Afterwards, crude extracts were purified by using C18 chromatography cartridges (Sep-Pack, Waters, Milford) to remove sugars and organic acids that could interfere in the total polyphenol content analysis (Li, Smith & Hossain, 2006b). Purified extracts were stored at -20ºC 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 60ºC 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

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

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

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

2.4.4. Total antioxidant activity (TAA)

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

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

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

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

2.5. Statistical analysis

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

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

where Y is the studied response and β_0, β_i, β_{ii} and β_{ij} are the independent, linear, quadratic and interaction coefficients, respectively. Coefficients of determination (R^2) were obtained for each fit.

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

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

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.

3. Results and discussion

3.1 Conventional solid-liquid extraction.

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

Naringin (18-28 mg/g dw) was by far the most abundant flavonoid independently of the experimental conditions used. Hesperidin (0.23-0.74 mg/g dw) and narirutin (0.28-0.70 mg/g dw) were the following most abundant flavonoids. These results are in agreement with those reported by other authors on the main flavonoids in grapefruit (Xu, Ye, Chen & Liu, 2007; Zhang et al., 2011; Goulas & Mangaris, 2012) 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 > neoeritrocin=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, Kriaa, 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 ºC 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=48ºC, t=32 min) gave the lowest TPC
324 while runs 1 and 4 (EtC=0.5 g/g (50 g/100 g), T=70ºC, t=32 min and EtC=0.5 g/g (50
325 g/100 g), T=48ºC, 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=48ºC, 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=48ºC, EtC=0.5 g/g (50 g/100 g), T=32 min) gave a TPC value of 71.0±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,

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

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

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

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

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

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

On average, TPC and TAA values in USE were about 1.7 folds the value obtained in CE in both cases, despite the shorter times of extraction in USE. This is in agreement with results obtained by several authors in the extraction of polyphenols from different fruits and vegetables (d'Alessandro et al., 2012; Da Porto et al., 2013; Khan, Abert-Vian, Fabiano-Tixier, Dangles & Chemat, 2010). The effect of the ultrasounds is attributed to their interaction with the plant material, altering its physical and chemical properties, and to the cavitational 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. Neoeritrocin, neohesperin and tangeritin 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=69ºC, EtC=0.3
399 g/g (30 g/100 g) and t=190 min; USE: T=25ºC, EtC=0.4 g/g (40 g/100 g) and t=55 min),
400 it is noticeable that for the USE higher predicted extraction yields are obtained (56.0 vs.
401 80.0 mg GAE/g dw, 23.5 vs. 38.3 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=25ºC and t=3 min, leading to expected response variables of TPC=69.7 mg

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

This selection was validated carrying out the extraction under the new experimental conditions (T=25ºC, EtC=0 and t=3 min). Experimental results were satisfactory, being TPC slightly higher than expected (108%), and TAA slightly lower (91%). Regarding the flavonoid analysis, it was found the same trend observed in the previous CE and USE treatments: (naringin (29 mg/g dw)>>hesperidin (0.82 mg/g dw)=narirutin (0.74 mg/g dw)>neoeritrocin (0.17 mg/g dw)=neohesperidin (0.11 mg/g dw)>tangeritin (0.017 mg/g dw)).

4. Conclusions

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

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

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

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

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List of symbols

ANOVA	analysis of variance
CE	conventional solid/liquid extraction
dw	dried weight
D	desirability function
DPPH*	2,2-diphenil-1-picrilhydracyl radical
EtC	ethanol concentration (ethanol weight/solution weight (g/g))
GAE	gallic acid equivalents
R^2	coefficient of determination
s_{CP}	standard deviation of center points
s_p	pooled standard deviation
T	temperature
t	time
TAA	total antioxidant activity
TPC	total polyphenols content
USE	ultrasound assisted solid/liquid extraction
$\bar{X}_{CP}$	mean of center points

Greek symbols

461 β_0 independent coefficient

462 β_i linear coefficient

463 β_{ii} quadratic coefficient

464 β_{ij} interaction coefficient

465

466 **References**

467

468 Amendola, D, De Faveri, D.M., Spigno, G. (2010) Grape marc phenolics: Extraction
469 kinetics, quality and stability of extracts, *Journal of Food Engineering 97*, 384-392.

470 Arnous, A., Makris, D.P., Kefalas, P. (2002) Correlation of pigment and flavanol
471 content with antioxidant properties in selected aged regional wines from Greece,
472 *Journal of Food Composition and Analysis 15*, 655-665.

473 Baç, D. and Boyaci, I.D. (2007) Modeling and optimization I: Usability of response
474 surface methodology, *Journal of Food Engineering 78*, 836-845.

475 Bocco, A., Cuvelier, M.E., Richard, H., Berset, C. (1998) Antioxidant activity and
476 phenolic composition of citrus peel and seed extract, *Journal of Agricultural and*
477 *Food Chemistry 46*, 2123-2129.

478 Chemat, F., Huma, Z., Khan, K.M. (2011) Applications of ultrasound in food
479 technology: processing, preservation and extraction. *Ultrasonics Sonochemistry 18*,
480 813-835.

481 d'Alessandro, L.G., Kriaa, K., Nikov, L., Dimitrov, K. (2012) Ultrasound assisted
482 extraction of polyphenols from black chokeberry, *Separation and Purification*
483 *Technology 93*, 42-47.

484 d'Alessandro, L.G., Dimitrov, K., Vauchel, P., Nikov, J. (2014) Kinetics of ultrasound
485 assisted extraction of anthocyanins from Aronia melanocarpa (black chokeberry)
486 wastes, *Chemical Engineering Research and Design 92*, 1818–1826

Da Porto, C., Porretto, E., Decorti, D. (2013) Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds, *Ultrasonic Sonochemistry 20*, 1076-1080.

Di Mauro, A., Fallico, B., Passerini, A., Maccarone, E. (2000) Waste water from Citrus processing as a source of hesperidin by concentration on styrene-divinylbenzene resin, *Journal of Agricultural and Food Chemistry 48*, 2291-2295.

Galanakis, C.M. (2012) Recovery of high-added value components from food wastes: conventional, emerging technologies and commercialized applications, *Trends in Food Science and Technology 26*, 68-87.

Giannuzzo, A.N., Boggetti, H.J., Nazareno, M.A., Mishima, H.T. (2003) Supercritical fluid extraction of naringin from the peel of *Citrus paradise*, *Phytochemical Analysis 14*, 221-223.

Girennavar, B., Jayaprakasha, G.K., Patil, B.S. (2008) Variation of bioactive furocoumarins and flavonoids in different varieties of grapefruits and pummelo, *European Food Research Technology 226*, 1269-1275.

Goulas, V., Manganaris, G.A. (2012) Exploring the phytochemical content and the antioxidant potential of *Citrus* fruits grown in Cyprus, *Food Chemistry 131*, 39-47.

Guo, C., Yang, J., Wei, J., Li, Y., Xu, J., Jiang, Y. (2003) Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay, *Nutrition Research 23*, 1719-1726.

Jogleker, A.M., May, A.T. (1999) Product excellence through experiment design, In: Food product development. Pp. 211-230, Aspen Publishers

Khan, M.K., Abert-Vian, M., Fabiano-Tixier, A.S., Dangles, O., Chemat, F. (2010) Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel, *Food Chemistry 119*, 851-858.

512 Ko, M.J., Cheigh, C.I., Chung, M.S. (2014) Relationship analysis between flavonoids
513 structure and subcritical water extraction (SWE), *Food Chemistry 143*, 147-155.
514 Kosseva, M.R. (2013) Functional food and nutraceuticals derived from food industry
515 wastes, In: Food Industry Wastes 103-120, Academic Press
516 Li, B.B., Smith, B., Hossain, Md.M. (2006a) Extraction of phenolics from citrus peels.
517 II. Enzyme-assisted extraction method, *Separation and Purification Technology 48*,
518 189-196.
519 Li, B.B., Smith, B., Hossain, Md.M. (2006b) Extraction of phenolics from citrus peels.
520 I. Solvent extraction method, *Separation and Purification Technology 48*, 182-188.
521 Librán, C.M., Mayor, L, Garcia-Castello, E.M. Vidal-Brotons, D. (2013) Polyphenol
522 extraction from grape wastes: Solvent and pH effect, *Agricultural Sciences 4*, 56-62.
523 Monier, V., Mudgal, S., Escalon, V., O'Connor, C., Gibon, T., Anderson, G., …,
524 Morton, G. (2010) Preparatory Study on Food Waste Across EU 27. Contract
525 07.0307/2009/540024/ser/g4. Final report. European Commission DG ENV.
526 Directorate C-Industry.
527 Montgomery, D.C. (2001). Design and Analysis of Experiments, 5th edn. Pp. 435–460.
528 New York: John Wiley & Sons, Inc
529 Octave, S., Thomas, D. (2009) Biorefinery: Toward an industrial metabolism,
530 *Biochimie 91*, 654-664.
531 Patil, B.S., Jayaprakasha, G.K., Chidambara, M.K.N., Vikram, A. (2009) Bioactive
532 compounds: historical perspectives, opportunities and challenges, *Journal of*
533 *Agricultural and Food Chemistry 57*, 8142-8160.
534 Pinelo, M., Rubilar, M., Sineiro, J., Núñez, M.J. (2004) Extraction of antioxidant
535 phenolics from almond hulls (*Prunus amygdalus*) and pine sawdust (*Pinus pinaster*),
536 *Food Chemistry 85*, 267-273.

Rodríguez Amado, I., Franco, D., Sánchez, M., Zapata, C., Vázquez, J.A. (2014) Optimisation of antioxidant extraction from *Solanum tuberosum* potato peel waste by surface response methodology, *Food Chemistry 165*, 290-299.

Roussis, I.G., Lambropoulos, I., Tzimas, P., Gkoulioti, A., Marinos, V., Tsoupeis, D., Boutaris, I. (2008) Antioxidant activities of some Greek wines and wine phenolic extracts, *Journal of Food Composition and Analysis 21*, 614-621.

Turkmen, N., Sari, F., Velioglu, Y.S. (2006) Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods, *Food Chemistry 99*, 835-841.

Vilkhu, K., Mawon, R., Simons, L., Bates, D. (2008) Applications and opportunities for ultrasound assisted extraction in the food industry-A review, *Innovative Food Science and Emerging Technologies 9*, 161-169.

Wang, Z., Shang, Q., Wang, W., Feng, X. (2011) Microwave-assisted extraction and liquid chromatography/mass spectrometry anaysis of flavonoids from grapefruit peel, *Journal of Food Process Engineering 34*, 844-859.

Xu, G., Ye, X., Chen, J., Liu, D. (2007) Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract, *Journal of Agricultural and Food Chemistry 55*, 330-335.

Yang, L., Jiang, J.G., Li, W.F., Chen, J., Wang, D.Y., Zhu, L. (2009). Optimum extraction process of polyphenols from the bark of *Phyllanthus emblica L.* based on the response surface methodology. *Journal of Separation Science 32 (9*), 1437-1444.

Zhang, M., Duan, C., Zang, Y., Huang, Z., Liu, G. (2011) The flavonoid composition of flavedo and juice from the pummelo cultivar (*Citrus grandis* (L.) Osbeck) and the grapefruit cultivar (*Citrus paradisi*) from China, *Food Chemistry 129*, 1530-1536.

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Table 1. Central composite design for the conventional and ultrasound assisted solid-liquid extraction of flavonoids from grapefruit peel. Coded and actual variables.

	Coded variables			Actual variables			
				Ethanol concentration	Temperature	Time (min)	
	X_1	X_2	X_3	(g/100 g)	(ºC)		
Run No.						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

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

Run No.	EtOH (g/100 g)	Temp. ºC	Time min	TPC[a,c] mg GAE[d]/g dw	TAA[b,c] mmol trolox/g dw	Neohesperidin mg/g dw	Neoeritrocin 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

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

Table 3. Regression coefficients of the fitted polynomial equations (Eq. 2) for total polyphenol content and total antioxidant activity as a function of the ethanol concentration, temperature and time. Conventional and ultrasound assisted extractions of flavonoids from grapefruit wastes.

Regression coefficients[a]	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												
β_0	82.7453			96.9826			12.5039			39.1254		
Linear												
β_1	-0.5670	25.29	0.0003[b]	0.6831	6.73	0.0340[b]	0.3078	26.43	0.0006[b]	0.0840	19.41	0.0017[b]
β_2	-1.6211	8.46	0.0127[b]	-1.3107	0.01	0.9316	-0.1219	0.98	0.3492	-0.1860	0.89	0.3701
β_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												
β_{11}	-0.0011	0.22	0.6517	-0.0107	29.15	0.0005[b]	-0.0044	16.08	0.0031[b]	-0.0039	27.55	0.0005[b]
β_{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
β_{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												
β_{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
β_{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
β_{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		

[a]where 0 = intercept; 1 = Ethanol concentration (g/100 g). 2 = temperature (ºC), 3 = time (min), [b]significant at α=0.05

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

Run No.	EtOH conc. (g/100 g)	Temp ºC	Time min	TPC[a,c] mg[d] GAE/g dw	TAA[b,c] mmol trolox/g dw	Neohesperidin mg/g dw	Neoeritrocin 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

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

Figure 1.

Coventional solid-liquid extraction of flavonoids from grapefruit peels: (a) Standarized Pareto chart for total polyphenol content (TPC) and total antioxidant activity (TAA); (b) TPC response surface at t=190 min. where dots represent experimental data; (c) TAA response surface at t=190 min; (d) Desirability function chart at t=190 min, where the black point indicates the coordinates of the optimized conditions (EtC=30 g/100 g (0.3 g/g); T=69ºC ; t=190 min).

Figure 2.

Ultrasound assisted solid-liquid extraction of flavonoids from grapefruit peels: (a) Standarized Pareto chart for total polyphenol content (TPC) and total antioxidant activity (TAA); (b) TPC response surface at t=55 min. where dots represent experimental data; (c) TAA response surface at t=55 min; (d) Desirability function chart at t=55 min, where the black point indicates the coordinates of the optimized conditions (EtC=40 g/100 g (0.4 g/g); T=25ºC ; t=55 min).

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606 FIGURE 1

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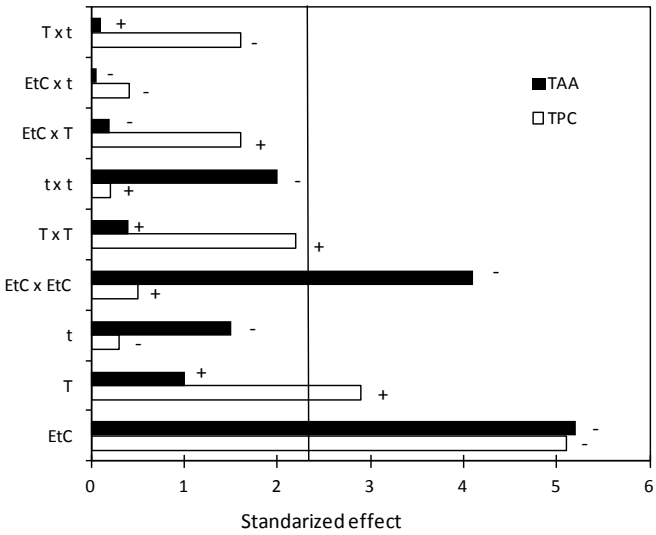

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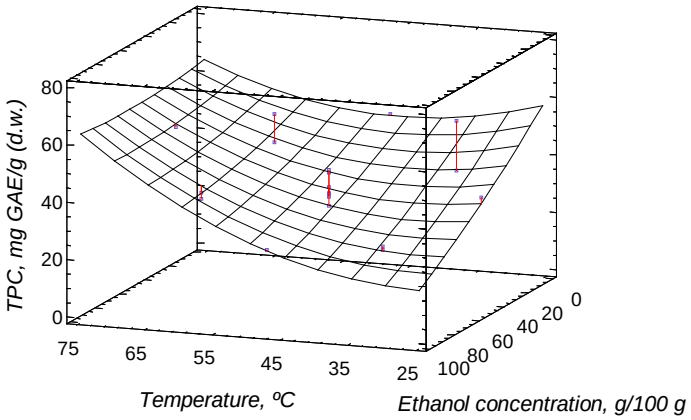

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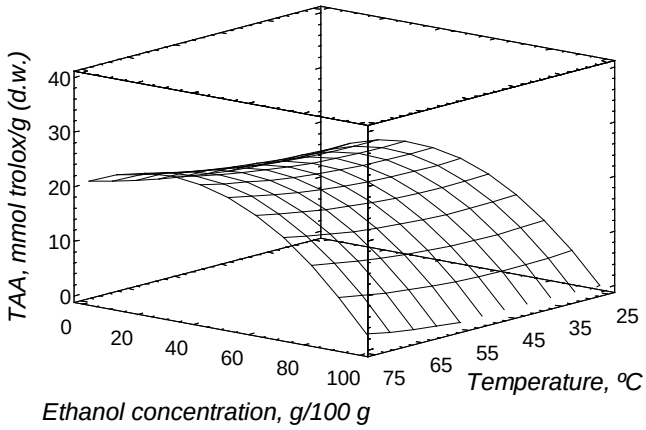

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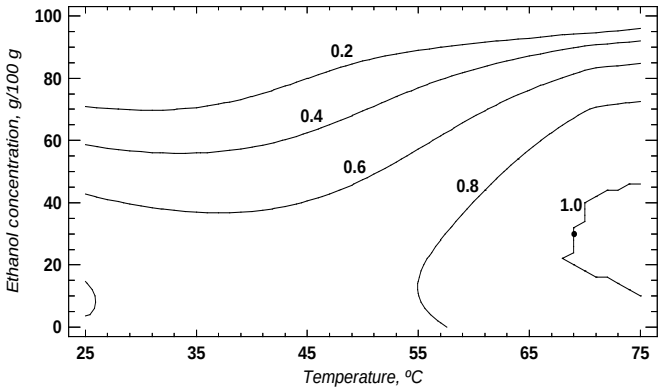

(d)

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614 FIGURE 2

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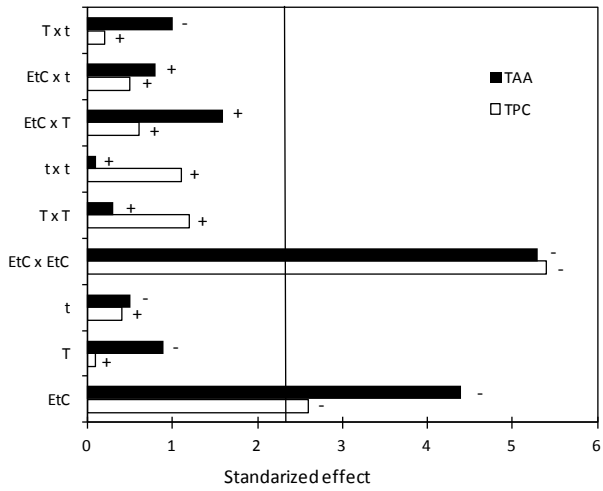

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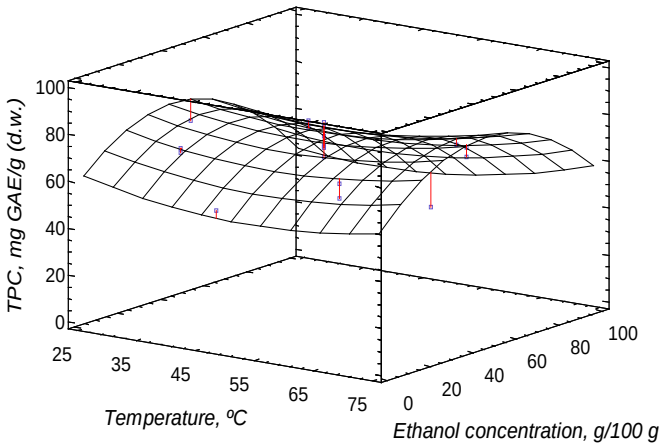

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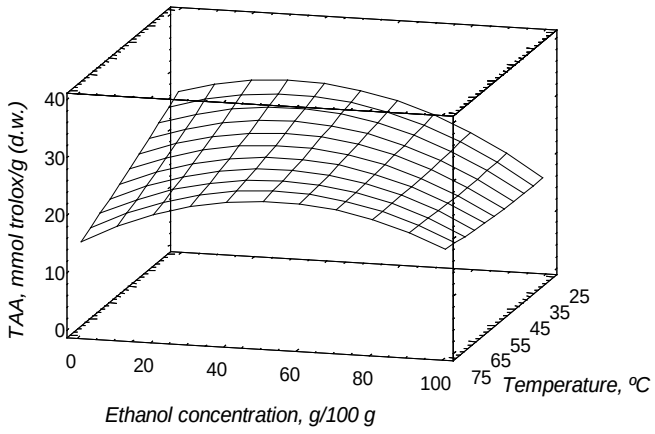

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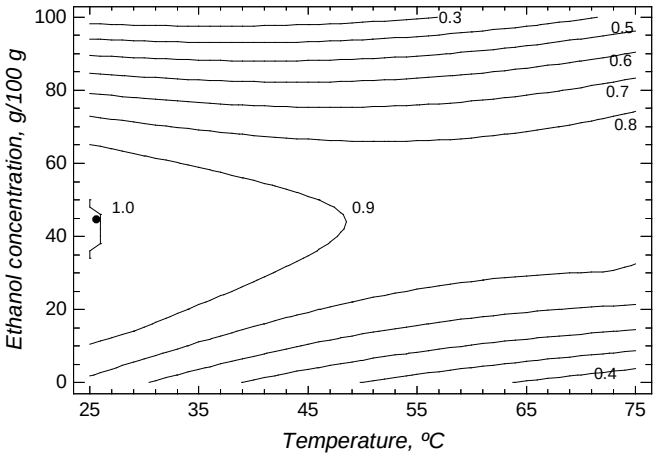

(d)

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