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Analytical Methods

Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel

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ABSTRACT

The present study reports on the extraction of polyphenols especially flavanones from orange (*Citrus sinensis* L.) peel by using ethanol as a food grade solvent. After a preliminary study showing that the best yield of extraction was reached for a particle size of 2 cm^2, a response surface methodology (RSM) was launched to investigate the influence of process variables on the ultrasound-assisted extraction (UAE) followed by a central composite design (CCD) approach. The statistical analysis revealed that the optimised conditions were a temperature of 40 °C, a sonication power of 150 W and a 4:1 (v/v) ethanol:water ratio. The high total phenolic content (275.8 mg of gallic acid equivalent/100 g FW), flavanone concentrations (70.3 mg of naringin and 205.2 mg of hesperidin/100 g FW) and extraction yield (10.9 %) obtained from optimised UAE proved its efficiency when compared with the conventional method. Furthermore, the antioxidant activity determined by the DPPH and ORAC tests confirmed the suitability of UAE for the preparation of antioxidant-rich plant extracts.

1. Introduction

Epidemiological studies have suggested the beneficial effects of citrus fruits (rich in flavanones) against many degenerative diseases like cardiovascular diseases and some cancers (Benavente-Garcia, Castillo, Marin, Ortuno, & Rio, 1997; Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). These positive influences on human health has significantly increased the citrus consumption in the last few years and it is continuously increasing with an estimated world production of citrus fruits up to 72 million tons in the session 2007–2008, among which the major commercially important orange fruits accounts for about 45 million tons (USDA, 2008). The domestic and industrial use of these large quantities of citrus fruits, especially for the production of juice, results in the accumulation of high amounts of by-products such as peel, seed, cell and membrane residues which account for about half of the fruit weight. These by-products can be used for the production of molasses, pectins, essential oils, limonene and cattle feed (Bocco, Cuvelier, Richard, & Berset, 1998; Jeong et al., 2004; Li, Smith, & Hossain, 2006a, 2006b). In addition, citrus by-products are a good source of phenolic compounds, especially the characteristic flavanone glycosides which mainly include naringin, hesperidin, narirutin, and neohesperidin. Currently, their extraction from citrus peels has attracted considerable scientific interest to use them as natural antioxidants mainly in foods to prevent the rancidity and oxidation of lipids (Anagnostopoulou, Kefalas, Papageorgiou, Assimopoulou, & Boskou, 2006; Peschel et al., 2006; Zia-ur-Rehman, 2006). Indeed, in recent years, a lot of research has focused on plants and their by-products to extract natural and low-cost antioxidants that can replace synthetic additives such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which might be liver-damaging, carcinogenic (Ak & Gülçin, 2008) and more generally toxic (Moure et al., 2001).

Up to now, several conventional extraction techniques have been reported for the extraction of phenols from citrus peels like solvent extraction (Anagnostopoulou et al., 2006; Jeong et al., 2004; Li et al., 2006a; Manthey & Grohmann, 1996; Xu, Ye, Chen, & Liu, 2007; Zia-ur-Rehman, 2006), hot water extraction (Xu et al., 2008), alkaline extraction (Bocco et al., 1998; Curto, Tripodo, Leuzzi, Giuffrè, & Vaccarino, 1992), resin-based extraction (Calvarano, Postorino, Gionfriddo, Calvarano, & Bovalo, 1996; Kim, Kim, Lee, & Kim, 2007), enzyme-assisted extraction (Li et al., 2006b), electron beam- and γ-irradiation-based extractions (Kim, Lee, Lee, Nam, & Lee, 2008; Oufedjikh, Mahrouz, Amiot, & Lacroix, 2000) and supercritical fluid extraction (Giannuzzo, Boggetti, Nazareno, & Mishima, 2003). These conventional or more innovative extraction techniques may either cause the degradation of the targeted compounds due to high temperature and long extraction times as in solvent extractions, or pose some health-related risks due to the unawareness of safety criteria during irradiation.

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Furthermore, enzyme-assisted extraction is limited due to problems of enzyme denaturation. With the development of the "Green Chemistry" concept during the last few years, environment-friendly techniques are becoming more and more attractive. The extraction of bioactive compounds under ultrasound irradiation (20–100 kHz) is one of the upcoming extraction techniques that can offer high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and temperature and lower energy input (Chemat, Tomao, & Virot, 2008).

During sonication, the cavitation process causes the swelling of cells or the breakdown of cell walls, which allow high diffusion rates across the cell wall in the first case or a simple washing-out of the cell contents in the second (Vinatoru, 2001). Besides the solvent, temperature and pressure, better recoveries of cell contents can be obtained by optimising ultrasound application factors including frequency, sonication power and time, as well as ultrasonic wave distribution (Wang & Weller, 2006). Optimisation of ultrasound-assisted extraction (UAE) has been described recently to extract hesperidin from Penggan (*Citrus reticulata*) peel (Ma, Chen, Liu, & Ye, 2008a), phenolic acids and flavanone glycosides from Satsuma Mandarin (*Citrus unshiu* Marc) peel (Ma, Chen, Liu, & Ye, 2009; Ma et al., 2008b) and total phenolic contents from Penggan peel (Ma, Chen, Liu, & Ye, 2008a) (see Table 1). In these works, methanol came up as a suitable extraction solvent to reach good yields of the above-mentioned phenolic compounds. However, environmentally benign and non-toxic food grade organic solvents like ethanol, *n*-butanol and isopropanol are recommended by the US Food and Drug Administration for extraction purposes (Bartnick, Mohler, & Houlihan, 2006).

A literature search did not yield any reference about earlier reports on the UAE of phenolic compounds from orange peels by using food grade solvents. The objective of this work is to outline the potentiality of UAE in the fast preparation of extracts rich in polyphenols (especially flavanone glycosides) from orange peels in good yields. Several parameters that could potentially affect the extraction efficiency were evaluated and optimised using a statistical experimental design approach. Finally, the optimised UAE results obtained were compared with those achieved by using a conventional extraction method.

2. Materials and methods

2.1. Plant material

About 10 kg of orange (*Citrus sinensis* L. Osbeck from Valencia late cultivar, Spain) peels after juice extraction were collected locally from a citrus juice industry (Vaucluse, France). They were stored in a freezer at −20 °C.

2.2. Chemicals

The solvents used were of analytical grade and supplied by VWR International (Darmstadt, Germany). Flavanone glycosides (naringin, hesperidin) were purchased from Extrasynthese (Genay, France), caffeic acid from Sigma–Aldrich (Steinhaus, Germany), and trolox from Acros Organics (Slangerup, Denmark). DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2′-azobis (2-methyl)propionamidine dihydrochloride) and fluoresceine were obtained from Alfa Aesar (Karlsruhe, Germany), Sigma–Aldrich (Steinhaus, Germany) and Acros Organics (Morris Plains, USA), respectively.

2.3. Instrumentation

2.3.1. Sonication apparatus

Ultrasound-assisted extraction (UAE) was performed with a PEX 3 Sonifier (R.E.U.S., Contes, France) composed of an inox jug having 23 × 13.7 cm internal dimensions with a maximal capacity of 3 L, and a transducer, in the base of jug, operating at a frequency of 25 kHz with maximum input power of 150 W. The double layered mantle allowed us to control the temperature of the medium by cooling/heating systems. The output power of the generator is 150 W while the power dissipated in the medium is about 60 W, as measured by calorimetry. The detailed diagram of the apparatus has shown in the Fig. 1.

2.3.2. HPLC analysis

HPLC analyses were performed using a Waters (Milford, MA) HPLC system consisting of a Waters 600E pump, a Waters 717 autosampler, a Waters 2996 photodiode array detector. The HPLC pumps, autosampler, column temperature, and diode array system were monitored and controlled by using Waters Empower 2 Chromatography Data software program. The wavelength used for the quantification of the flavanones glycosides with the diode detector was 280 nm. The chromatographic separation was carried out on a Purospher Star RP-18 end-capped column (250 × 4 mm I.D.; 5 μm particle size from VWR), with a RP-18 guard column (4 × 4 mm I.D.; 5 μm particle size also from VWR). The end-capped column and guard column were held at 37 °C and the flow rate was set at 1 mL/min. The mobile phase consisted of two solvents: 0.5% acetic acid (A) and 100% acetonitrile (B). The solvent gradient in volume ratios was as follows: 10–30% B over 20 min. The solvent gradient was increased to 35% B at 25 min and it was maintained at 35% B for 5 min. The injection volume was 20 μL. Analyses were performed at least three times and only mean values were reported. Quantification was carried out by using the external standard method and the final concentrations were calculated in mg/100 g FW.

Table 1
Recent publications on the extraction of polyphenols under ultrasound irradiation.

Plant material	Analytes	Comments	References
Satsuma Mandarin *Citrus unshiu* Marc	Phenolic acids (PA)	UAE time = 10–40 min; maceration for 8 h for similar yields of PA	Ma et al. (2009)
Du Zhong Ye *Folium eucommiae*	Flavonoids	UAE was found more efficient than heating, microwave- and enzyme-assisted extractions	Huang, Xue, Niu, Jia, and Wang (2009)
Wheat bran *Triticum aestivum*	Phenolics-rich heteroxylans	Extraction time reduced from 60 min (conventional extraction) to 5 min (UAE)	Hromádková, Košťálová, and Ebringerová (2008)
Penggan *C. reticulata*	Hesperidin	Comparable yields with UAE but less degradation of hesperidin compared with soxhlet extraction	Ma et al. (2008c)
Satsuma mandarin *C. unshiu* Marc	Phenolic acids and flavanone glycosides	Increase in polyphenol content and antioxidant activity of extracts obtained by UAE in comparison with maceration	Ma et al. (2008b)
Penggan *C. reticulata*	Total phenolic content (TPC)	TPC increased on increasing irradiation time and temperature	Ma et al. (2008a)
Winged burning bush *Euonymus alatus*	Flavonols rutin and quercetin	UAE efficiency monitored by microscopy	Yang and Zhang (2008)

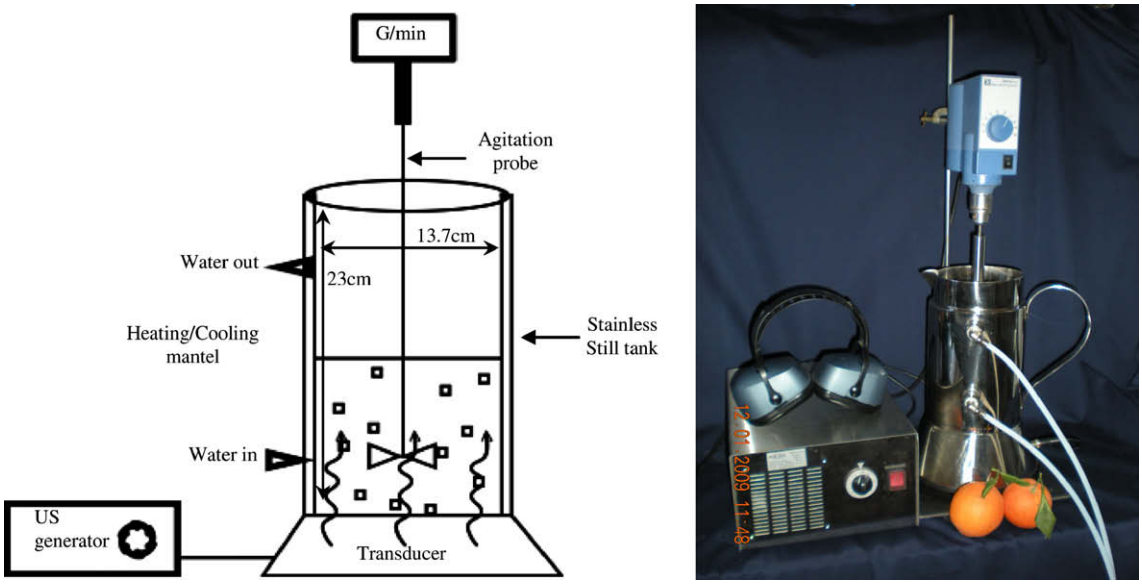

Fig. 1. Sonication apparatus used for UAE.

2.3.3. Spectrophotometers

Absorbance measurements were carried out on a Spectronic GENESYS 5 UV–Visible spectrophotometer (wavelength range: 200–1100 nm) equipped with a eight-position multicell holder. Measurements of fluorescence intensity were carried out on a SPEX-Fluoromax 2 spectrofluorimeter from Jobin Yvon.

2.4. Extraction procedure

A comparative study has been conducted between the conventional and ultrasound-assisted techniques after the optimisation of the latter. *UAE*: in experiments aimed at optimising the extraction temperature, ultrasound power, and ethanol percentage, orange peels (0.25 g/mL) were sonicated in the solvent (ethanol–water mixture) for 30 min. The optimal parameters were further used to investigate the extraction time required for maximal yield. *Solvent extraction* (*SE*): a control extraction was run by using the temperature and ethanol percentage that were found optimal for UAE.

2.5. Particle size study

A series of five experiments with five different particle sizes (0.5, 1.0, 1.5, 2.0 and 2.5 cm^2) was carried out by using the conventional solvent extraction procedure with central point conditions (25 °C, 1:1 ethanol–water solution, stirring, 30 min). Peel particles having a thickness of about 0.5 cm were cut out randomly with help of calibrated steel cubes.

2.6. Total phenolic content (TPC)

The TPC of samples was measured with a kit (SEPPAL (Isiteclab), France) especially suitable for TPC measurement of foods and drinks. This kit includes reagent A (modified Folin–Ciocalteu reagent), reagent B (alkaline buffer) and a gallic acid solution (3 g/L). A small volume (20 μL) of H_2O (blank), gallic acid solution (standard) or the extract (sample) was mixed with reagent A (2 mL). After 1 min, 1 mL of reagent B was added to each sample. The mixtures were allowed to stand for 30 min in the dark at room temperature. Then, their absorbance was measured at 760 nm. TPCs were calculated by using the following formulae: TPC = 3 × (sample absorbance − blank absorbance)/(standard absorbance − blank absorbance). TPC measurements were performed thrice and mean values, expressed as mg gallic acid/100 g of fresh weight (mg GA/100 g FW), were reported.

2.7. Yield determination

Ethanol was removed from the extracts by evaporation under vacuum at 40 °C on a rotary evaporator. Then, the samples were frozen and lyophilised to remove water. Finally, the yield of each extract was calculated from its weight and expressed in percentage.

2.8. Design of experiment

Box-Wilson design, also called central composite design (CCD), is used to achieve maximal information about the process from a minimal number of possible experiments. The type of CCD used in this study was central composite face-centred (CCF) experimental design to determine the optimal conditions of UAE. The application of a CCF design is a convenient way to optimise a process with three levels (−1, 0 and +1) for each factor. In this design, the star points are at the centre of each face of the factorial space, thus $\pm\alpha = \pm 1$. This design is needed to evaluate the effects and interactions of three independent variables, namely temperature (°C) (*X*1), power (W) (*X*2) and ethanol:water ratio (%v/v) (*X*3). The coded levels and the natural values of the factors used in this experimental design are shown in Table 2 in parallel. A total of 20 different combinations, including six replicates of centre point, each designated by the coded value 0, were chosen in random order according to a CCF configuration for three factors. The selected optimisation parameters were TPC after 30 min (mg gallic acid/100 g fresh weight) (*Y*1), naringin concentration (mg/100 g FW) (*Y*2), hesperidin concentration (mg/100 g FW) (*Y*3), yield of extracts (%) (*Y*4) and extraction rate constant (min^{-1}) (*Y*5).

The experimental designs used were constructed and the experimental results were processed by using the software STATGRAPHICS PLUS (Version 5.1, Statistical Graphics Corporation, Rockville, USA, 2000). An analysis of variance (ANOVA) with 95% confidence level was then carried out for each response variable in order to test the model significance and suitability. The *F*-value in ANOVA

is the ratio of mean square error to the pure error obtained from the replicates at design centre and the *P*-value defines the significance of the different variables. A description of significant effects obtained from ANOVA for TPC (30 min) was presented by a Standardised Pareto Chart.

2.9. Kinetic studies

2.9.1. Extraction rate constant (k)

During each extraction process, uptake of 1 mL from the mixture was performed at 5, 10, 20 and 30 min to determine the corresponding TPC values. Assuming a first-order accumulation of total phenols in solution (Fig. 4), we can write:

$$TPC_t = TPC_\infty(1 - e^{-kt})$$

TPC_t: TPC value at time t, TPC_∞: final TPC value (determined at $t = 8$ h), k: apparent first-order rate constant of extraction. Thus, from the linear plots of $-\mathrm{Ln}\,(1 - (TPC_t/TPC_\infty))$ against time (correlation coefficient in the range 0.90–0.99), the k values could be determined.

2.9.2. Activation energy (E_a)

From the Arrhenius equation, the activation energies for total phenol extraction by UAE and SE were determined from plots of $\mathrm{Ln}\,k$ against $1/T$, where T is the absolute temperature (283, 298 and 313 K).

2.10. Antioxidant tests

As there is no standardised method to evaluate the antioxidant potential of foods and biological systems, it is recommended to evaluate the antioxidant activity by various methods (Frankel & Meyer, 2000).

2.10.1. DPPH assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable highly coloured free radical that can abstract labile hydrogen atoms from phenolic antioxidants with concomitant formation of a colourless hydrazine (DPPH-H) (Diouf, Stevanovic, & Cloutier, 2009). The free radical-scavenging activity (FRSA) of an extract can be expressed as the percentage of DPPH reduced by a given amount of extract. The FRSA of the extracts was evaluated according to the method described by Mimica-Dukic, Bozin, Sokovic, and Simin (2004), with some modifications. The extract was dissolved in 50% (v/v) aqueous methanol with a final concentration of 5 g/L. A small volume (0.1 mL) of the extract solution was mixed with 2.0 mL of a 0.1 mM DPPH solution in MeOH and the mixture was left in the dark at room temperature for 60 min. The absorbance was measured at 517 nm. The total FRSA of each extract was expressed as the percentage of DPPH reduced and was calculated by the following equation: FRSA = 100 × (initial absorbance − final absorbance)/initial absorbance. The initial absorbance and final absorbance are the absorbance values of DPPH at time zero and after 60 min, respectively.

2.10.2. ORAC (oxygen radical absorbance capacity) assay

In this method, the hydrophilic peroxyl radicals (ROO·) generated by the thermal decomposition of the diazo compound AAPH oxidise the fluorescent probe FL, thus causing a fluorescence quenching. Hence, inhibition of this quenching by an antioxidant is a measurement of its ability to reduce ROO· (Gomes, Fernandes, & Lima, 2005). The ORAC method employed was adapted from a method previously described by Ou, Hampsch-Woodill, and Prior (2001). All reagents were prepared in a 75 mM phosphate buffer at pH 7.4. Trolox (0–75 μM) was used as the standard. A mixture of the fluorescent probe fluorescein (FL, 2 mL of a 26 nM solution in phosphate buffer) and extract (15 μL of a 5 g/L solution in MeOH) was pre-incubated for 10 min at 37 °C. Then, 1 mL of a 664 mM AAPH solution in the phosphate buffer was added. The fluorescence intensity was measured every 2 min during 40 min with excitation and emission wavelengths set at 490 and 511 nm, respectively. Its decay refers to FL oxidation by the AAPH-derived peroxyl radicals. The ORAC value is calculated from the area under the curve expressing the quenching of FL fluorescence in the presence of the extract in comparison with curves constructed with known trolox concentrations. The measurements were taken in triplicate. The area under the curve (AUC) was calculated as $AUC = 1 + f_2/f_0 + f_4/f_0 \ldots + f_i/f_0$ where f_i is the fluorescence reading at time i (in s). The net AUC was obtained by subtracting the AUC of the blank (no antioxidant). The results were expressed as millimoles of trolox equivalents (TE) per gram of sample on a fresh weight basis (mmol TE/100 g FW). Both antioxidant tests (DPPH and ORAC) were performed at least three times for each extract and only mean values were reported.

3. Results and discussion

3.1. Influence of particle size

From previous studies (Cuoco, Mathe, Archier, Chemat, & Vieille-escazes, 2009; Garcia-Ayuso & Luque de Castro, 1999; Vilkhu, Mawson, Simons, & Bates, 2008; Wang & Weller, 2006), the particle size was considered one of the important factors that can affect the efficiency of polyphenol extraction from orange peels. Thus, preliminary experiments on orange peels of 0.5, 1.0, 1.5, 2.0 and 2.5 cm^2 gave final yields of 3.44%, 3.65%, 4.32%, 4.41% and 4.38%, respectively. A size of 2 cm^2 was selected as an optimum for our extraction experiments. The slightly lower yield observed with particles of smaller size could be due to the particles staying at the surface of the solvent during extraction, thereby limiting their exposition to ultrasonic waves.

3.2. Central composite design results

The coded and decoded values of independent variables and the responses obtained in the multivariate study for each experiment are shown in Table 2. In this second part of the study, the effect of temperature (°C) *X*1, ultrasonic power (W) *X*2 and ethanol:water ratio (% v/v) *X*3 on UAE of orange peel polyphenols in terms of TPC (mg GA/100 g FW) *Y*1, naringin concentration (mg/100 g FW) *Y*2 and hesperidin concentration (mg/100 g FW) *Y*3 was evaluated by response surface methodology. Identification of naringin and hesperidin was achieved by comparing their retention times and UV spectra with standards (Fig. 2). The yield (%) *Y*4 and the extraction rate constant (k) (min^{-1}) *Y*5 were also determined. ANOVA for TPC determination (30 min) gave a coefficient of determination (R^2) of 98.3%, which indicates a close agreement between experimental and predictive values. ANOVA data for TPC are also shown on a Pareto Chart (Fig. 3), which represents the significant effects of all variables (linear and quadratic) and their interactions. The length of the bars is proportional to the absolute magnitude of the estimated effects coefficients while the dashed line represents the minimal magnitude of statistically significant effects (95% of the confidence interval) with respect to response. It can be seen that ultrasound power has the most important influence on TPC followed by temperature, ethanol:water ratio, interaction of power and ethanol:water ratio, squared term of temperature and interaction of power and temperature. The lack of significance of the cross-product terms suggests the absence of interactions between variables in the studied range.

Table 2
Central composite design of three variables with their observed responses.

Exp. No.	Coded variables			Decoded variables			Responses				
	*X*1	*X*2	*X*3	T*	P*	E*	TPC 30 min (mg GA/100 g)	Naringin (mg/100 g)	Hesperidin (mg/100 g)	Yield (%)	Extraction rate constant (min^{-1})
1	0	0	0	25	100	50	185.493	36.193	119.290	7.8	0.0260
2	1	1	1	40	150	80	233.460	48.610	146.729	10.03	0.0402
3	0	0	0	25	100	50	185.068	32.721	112.853	7.77	0.0197
4	1	−1	1	40	50	80	197.646	33.347	113.332	8.09	0.0170
5	−1	−1	−1	10	50	20	121.259	17.831	71.692	6.27	0.0126
6	0	−1	0	25	50	50	162.480	28.247	93.183	6.97	0.0150
7	0	0	0	25	100	50	183.531	35.694	118.834	7.81	0.0215
8	0	0	0	25	100	50	185.994	33.295	113.186	7.76	0.0210
9	−1	1	1	10	150	80	187.276	34.007	117.123	7.93	0.0253
10	1	−1	−1	40	50	20	140.352	21.640	86.740	6.81	0.0152
11	0	0	−1	25	100	20	174.472	30.860	99.236	7.14	0.0153
12	0	0	1	25	100	80	192.352	32.260	110.866	7.96	0.0189
13	0	0	0	25	100	50	184.666	32.051	112.310	7.78	0.0208
14	−1	1	−1	10	150	20	169.918	29.345	96.847	7.09	0.0292
15	−1	0	0	10	100	20	159.217	29.531	98.749	6.89	0.0164
16	1	1	−1	40	150	20	225.302	36.469	124.489	9.59	0.0353
17	1	0	0	40	100	50	190.878	31.257	106.286	8.15	0.0186
18	−1	−1	1	10	50	80	155.258	24.561	87.903	6.86	0.0125
19	0	0	0	25	100	50	186.496	34.051	118.650	7.75	0.0211
20	0	1	0	25	150	50	213.188	35.146	119.252	8.93	0.0326

* $T = X1$ = temperature (°C); $P = X2$ = power (W); $E = X3$ = ethanol:water ratio (% v/v).

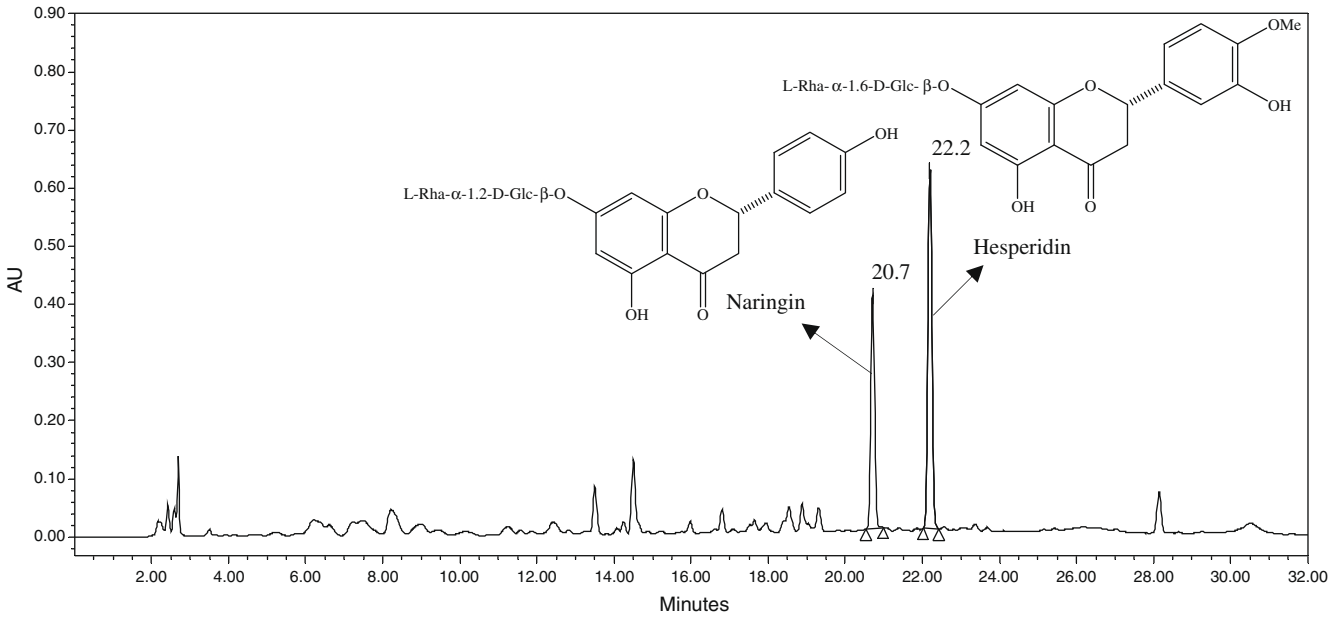

Fig. 2. HPLC analysis of an extract obtained by ultrasound-assisted extraction of orange peels.

The experimental data built after running 20 trials allowed us to fit all the responses as a function of temperature, power and ethanol:water ratio. The second-order polynomial equations of the response surfaces obtained are as follow:

$$Y1\ (\text{mg GA}/100\,\text{g}) = 184.782 + 19.471T + 25.2149P + 13.4689E - 9.09455T^2 + 5.01087TP + 1.76187TE + 3.69195P^2 - 8.22213PE - 0.730045E^2 \quad (1)$$

$$Y2\ (\text{mg}/100\,\text{g}) = 33.2623 + 3.6048T + 5.7951P + 3.664E - 1.7605T^2 + 1.1415TP + 1.557TE - 0.458P^2 - 0.20425PE - 0.5945E^2 \quad (2)$$

$$Y3\ (\text{mg}/100\,\text{g}) = 112.432 + 10.5262T + 15.159P + 9.6949E - 4.7805T^2 + 2.09637TP + 1.54313TE - 1.0805P^2 - 0.035875PE - 2.247E^2 \quad (3)$$

$$Y4\ (\%) = 7.72445 + 0.763T + 0.857P + 0.397E - 0.123636T^2 + 0.35375TP + 0.03625TE + 0.306364P^2 - 0.07375PE - 0.0936364E^2 \quad (4)$$

$$Y5\ (\text{min}^{-1}) = 0.0204645 + 0.00303T + 0.00903P + 0.00063E - 0.00113636T^2 + 0.0017375TP + 0.0013375TE + 0.00516364P^2 - 0.0000875PE - 0.00153636E^2 \quad (5)$$

where T is the temperature (°C), P the ultrasound power (W) and E the ethanol:water ratio (% v/v).

3.3. Optimal conditions

Response surface optimisation can be found depending on the three key variables, namely, temperature, power and ethanol:water ratio. The optimal conditions obtained from the first derivatives of the second-order polynomial equation were derived a second time. The derivatives were then equalled to 0 and solved in an equation system. The coded values obtained from these equations were thus decoded and rounded in order to be applied to the device. The obtained natural values corresponding to optimal conditions for each response were as follows: $Y1$ = 40 °C, 150 W, 80%; $Y2$ = 40 °C, 150 W, 80%; $Y3$ = 40 °C, 150 W, 80%; $Y4$ = 40 °C, 16 W, 80%; $Y5$ = 39 °C, 50 W, 69%. As expected and according to the response surfaces, the extraction efficiency in terms of TPC, naringin and hesperidin concentrations increases by increasing all the three factors. In all these responses, the optimal values were beyond the limits that we selected. Thus, the values finally selected correspond to the maximal values chosen to define the experimental domain (Lucchesi, Smadja, Bradshaw, Louw, & Chemat, 2007). On the basis of our principle responses ($Y1$, $Y2$, $Y3$), the temperature of 40 °C, ultrasound power of 150 W and ethanol:water ratio (v/v) of 80% were chosen as optimal values to go on with our experiments. A repeatability study was conducted by using these optimal conditions to assess the predictive ability of the models and the results were found in accordance with those obtained in the second trial {+1 (40 °C), +1 (150 W), +1 (80% ethanol)} of experimental design. Several recent investigations on the extraction of phenolic contents from citrus peel have also suggested operating conditions similar to those recommended in this study (Li et al., 2006a; Ma et al., 2008c, 2009).

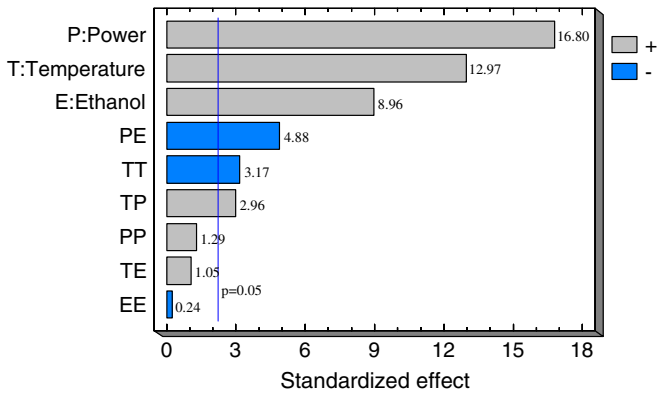

Fig. 3. Pareto Chart for total phenolic content (mg GA/100 g) at 30 min.

3.4. Comparison of UAE vs. SE

TPC extracted from orange peels by UAE (40 °C, 150 W, 80% ethanol, stirring) and SE (idem except sonication) is shown on Fig. 4. The TPC obtained by UAE during 15 min was significantly higher than by SE during 60 min. Due to mechanical effects on cell walls evidenced by scanning electron microscopy (Balachandran, Kentish, Mawson, & Ashokkumar, 2006; Li, Pordesimo, & Weiss, 2004), UAE permits higher extraction yields in shorter periods of time, thereby reducing the energy input.

The main flavanone glycosides found in orange (*C. sinensis*) are naringin and hesperidin, the latter being more abundant than the former (Wang, Chuang, & Hsu, 2008). Both were simultaneously titrated by HPLC from the samples obtained by UAE and SE after 60 min. The quantities of naringin and hesperidin from UAE (70.3 and 205.2 mg/100 g of fresh weight, respectively) were considerably higher than those obtained from SE (50.9 and 144.7 mg/100 g FW, respectively). No evidence for flavanone degradation under sonication could be found. Indeed, the ultrasonic degradation of phenols is typically slow in comparison with more volatile aromatics that diffuse more readily into the cavitation bubble for pyrolysis (Chowdhury & Viraraghavan, 2009). In addition, phenol degradation is favoured at higher frequencies (required for the generation of the hydroxyl radical by water homolysis) than the one selected in this work (20 kHz). The extraction yield is an important response factor for evaluating an extraction process. It was estimated to be 10.9% and 8.6% for UAE and SE, respectively. This is consistent with UAE having a potential to extract natural products in better yields than conventional techniques, not only at the lab-scale but also at the pilot-plant scale (Boonkird, Phisalaphong, & Phisalaphong, 2008).

Extraction of total phenols was found *ca.* 3 times as fast under ultrasounds (k = 0.10 (±0.01) min^{-1}) as in the conventional procedure (k = 0.03 (±0.01) min^{-1}). Consistently, the activation energy (6.34 kJ/mol for UAE vs. 34.21 kJ/mol for SE) is smaller. Similar kinetic effects were evidenced by Chemat, Lagha, Aitamar, Bartels, and Chemat (2004) for the UAE of caraway seeds in hexane.

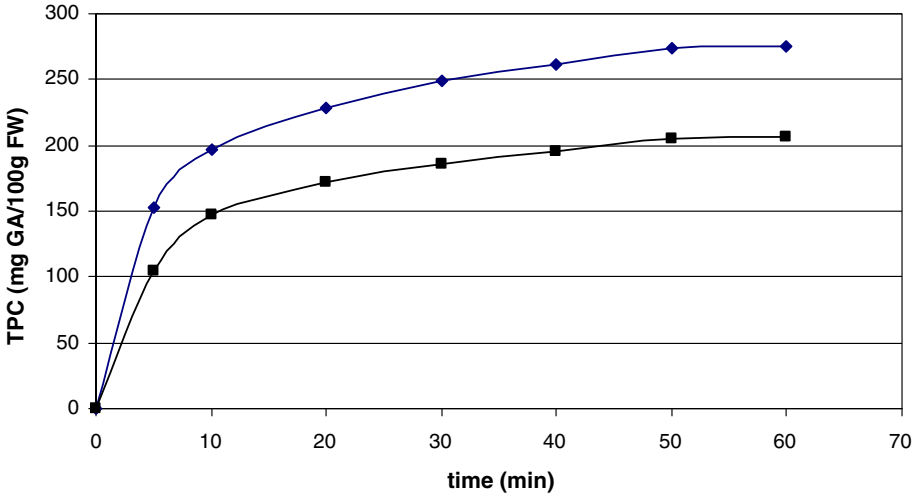

Fig. 4. Comparison of total phenolic contents (mg GA/100 g) from ultrasound-assisted extraction (UAE) —◆— and solvent extraction (SE) —■—.

3.5. Antioxidant capacities

UAE and SE were finally evaluated by comparing the antioxidant potential of the corresponding extracts. Phenolic antioxidants are typically able to quickly reduce reactive oxygen species (ROS) including free radicals, thereby protecting biomolecules (*e.g.*, polyunsaturated fatty acids) against oxidation (Dangles, 2006). The FRSA value was 54% and 42% for the extracts obtained by UAE and SE, respectively. The increase in FRSA observed with UAE, although modest, is in agreement with the higher total phenol concentration estimated by UAE and confirms the usual correlation between antioxidant activity and TPC (Anagnostopoulou et al., 2006; Ma et al., 2008b). The ORAC values were estimated to be 712 mmol TE/100 g FW and 509 mmol TE/100 g FW for the UAE and SE extracts, respectively.

While UAE during 60 min results in a 35–40% increase in TPC vs. SE, the FRSA estimated by the DPPH assay increases by less than 30% and the ORAC value by 40%. Hence, the ORAC assay appears more consistent with the increase in TPC than the DPPH radical assay. Indeed, the major orange flavanones naringenin and hesperetin are relatively weak antioxidants since they do not display a catechol group (1,2-dihydroxybenzene), which is the critical structural determinant of strong phenolic antioxidants (Goupy, Loonis, Dufour, & Dangles, 2003). As a consequence, they are expected to react very slowly with the stable DPPH radical. Thus, much more reactive radicals, such as the peroxyl radicals delivered in the ORAC test, are required to fully express the electron/H-donating activity of orange flavanones (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009). It must also be pointed out that nonphenolic antioxidants such as ascorbate (which is responsive to the test for TPC determination) can be partially responsible for the overall antioxidant activity of the extracts, especially in the DPPH assay where the flavanones are expected to make a minor contribution.

4. Conclusion

The UAE of phenolic antioxidants from orange peels with ethanol–water mixtures appeared very effective in comparison to conventional procedure. The results from CCD pointed out the sonication power as the most influential factor in the UAE process followed by temperature and ethanol:water ratio. Although the same volumes of solvent were used in both extraction processes, the duration of the ultrasound-assisted process and consequently the energy input were drastically reduced without affecting the overall yield. Hence, UAE can be called an 'environment-friendly' or 'green' technique. Overall, ultrasound-assisted extraction of polyphenols from abundant food by-products such as orange peels and by using food grade solvents has a strong potential of industrial development as an efficient and environment-friendly process for the preparation of extracts rich in natural antioxidants aimed at replacing synthetic antioxidants.

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