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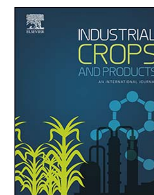

Optimization and comparison of maceration and microwave extraction systems for the production of phenolic compounds from *Juglans regia* L. for the valorization of walnut leaves

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ABSTRACT

This study compares maceration (ME) and microwave assisted (MAE) extractions aiming to maximize the extraction of valuable compounds from *Juglans regia* L. leaves. An experimental design, assisted by response surface methodology, was developed to optimize the variables of the ME and MAE systems (time, temperature and ethanol-water proportion). The responses used as criteria were the quantification by HPLC-DAD (3-*O*-caffeoylquinic acid, quercetin 3-*O*-glucoside and quercetin *O*-pentoside), the extraction yield and the spectrophotometric results of total phenolics and flavonols. The global optimum conditions were: 112.5 min, 61.3 °C and 50.4% of ethanol for ME; and 3.0 min, 107.5 °C and 67.9% of ethanol for MAE. The solid/liquid ratio effect was studied at the optimal values showing a decreasing linear relation until 140 g/L for all criteria assessed. This study contributes to the valorization of walnut leaves as source of valuable compounds (*e.g.*, quercetin) to be used as ingredients for the development of functional foods.

1. Introduction

There have been an increasing number of studies on extraction methodologies of secondary metabolites from plants, namely phenolic compounds, due to their bioactivity and manifest effects on oxidative processes related to several health diseases (Carocho and Ferreira, 2013). Some of these bioactive compounds are used as nutraceuticals due to their benefits in the prevention or treatment of health conditions (Bravo and Mateos, 2008). Moreover, they have been widely used in pharmaceuticals, functional foods and natural cosmetics (Martins et al., 2011).

Juglans regia L. (walnut) leaves have been applied in traditional medicine for topical and enteric use with different purposes (Carvalho and Morales, 2010; Guarrera, 2005) and several studies have reported the *in vitro* bioactivity of its extracts including antioxidant (Almeida et al., 2008; Carvalho et al., 2010; Pereira et al., 2007; Santos et al., 2013), antitumor (Santos et al., 2013), anti-inflammatory, and antiproliferative (Carvalho et al., 2010) properties. To demonstrate the bioactivity of the extracts, some *in vivo* models were also applied. Erdemoglu et al. (Erdemoglu et al., 2003) studied the oral administration of walnut leaves extracts with successful results on the anti-inflammatory activity using mice models inducing an antinociceptive action. The antidiabetic activity induced by walnut leaves extracts through rats models were also described in previous reports (Asgary et al., 2008; Mohammadi et al. 2012) inducing the reduction of blood sugar levels as well as the decrease of HbA_1C fraction. In addition, some of these studies refer the absence of cytotoxicity in porcine liver cells (Santos et al., 2013) or gastric damage in mice (Erdemoglu et al., 2003).

Previous studies (Santos et al., 2013) indicated that the alcoholic extracts of walnut leaves have a promising bioactivity and their chemical characterization showed that they are a rich source of phenolic compounds, being 3-*O*-caffeoylquinic acid, quercetin 3-*O*-glucoside and quercetin *O*-pentoside the main molecules present in *J. regia* leaves. Examples of the individual importance of these compounds can be found. 3-*O*-caffeoylquinic acid acts as a scavenger on O_2^- capture, exerting an inhibitory effect against oxidation of methyl linoleate (Nakatani et al., 2000). Quercetin aglycones (namely 3-*O*-glucoside) are found to play a critical role in the inhibition of human neutrophil

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elastase (so, immunomodulatory action in inflammatory responses) (Ryu et al., 2016) as well as in the cytotoxic protection against H_2O_2, leading to a decrease in the generation of reactive oxygen species (Shokoohinia et al., 2015). Thus, extracts rich in these compounds could potentially be included in several industries due to their versatility (*e.g.*, as ingredients in functional foods).

Generally, 3-*O*-caffeoylquinic acid is found in small amounts in natural matrices: 0.015 mg/g dw in *Vaccinium floribundum* Kunth berries (Prencipe et al., 2014); 0.76 and 0.89 mg/g dw in *Lonicera japonica* Thunb. flowers (Li et al., 2014); 6.41 mg/g dw in *J. regia* leaves (Santos et al., 2013), being the highest content (17.2 mg/g dw) reported by Orqueda et al. (Orqueda et al., 2017) on *Solanum betaceum* skin extract. On the other hand, quercetin 3-*O*-glucoside is frequently present in plant material in higher quantities: 33.40 mg/g dw in *Securigera securidaca* flowers (Ibrahim et al., 2015), 36.7 mg/g dw in *Dasiphora fruticosa* (L.) Rydb. (Tomczyk et al., 2012), 0.218 mg/g dw in *Vaccinium corymbosum* L. (Zorenc et al., 2017); 0.494 mg/g dw in *Salvia fruticosa* Mill. Leaves (Sarrou et al., 2016). However, the presence of quercetin *O*-pentoside is not so recurrent: 0.0133 mg/g dw on *Rosa canina* hips (Cunja et al., 2015), 5.04 mg/g dw in *J. regia* leaves (Santos et al., 2013), 0.101 mg/g fw in *V. floribundum* berries (Prencipe et al., 2014). More information can be found in Table 1.

The solid-liquid extraction of these compounds is usually carried out using organic solvents such as methanol, ethanol, acetone and ethyl acetate (Kerton, 2009). In particular, the most common solvents used by researchers are aqueous methanol and ethanol (Bravo and Mateos, 2008). The Food Drug and Administration (FDA) favours ethanol in the pharmaceutical and food industries due to its lower toxicity. In addition, this alcohol can be obtained from renewable sources and is safe for human consumption (Kerton, 2009).

Conventional systems such as Soxhlet and maceration (ME) require considerable amounts of solvents and are time-consuming processes. Thus, modern extraction techniques are being applied in the extraction of valuable compounds from natural sources. Microwave assisted extraction (MAE) is one of the most employed alternative extraction methods, commonly using mixtures of ethanol and water as solvent. Among the advantages reported for MAE are its lower extraction times and solvent consumption, when compared to conventional methods (Destandau et al., 2013; Routray and Orsat, 2012). Independently of the system applied on the extraction of target compounds from matrices, there are several process variables that require individual consideration due to the intrinsic nature and stability of these target compounds. Therefore, it is essential to identify the main variables involved, prior to the optimization process to maximize their responses, using the minimum time, energy and solvent consumption and designing the most cost-effective and profitable extraction system (Dai and Mumper, 2010). The most frequent form to carry out an optimization is by independently measuring the influence of each variable fixing all others. Nevertheless, the application of mathematical models such as the response surface methodology (RSM) is gaining visibility in the scientific community. The RSM design allows to optimize all the variables simultaneously and to predict the most efficient conditions. This is achieved by using second order polynomial models with interactions, that are able to describe and maximize the response criteria selected, based on the experimental range tested (Bezerra et al., 2008; Ferreira et al., 2007; Kalil and Maugeri, 2000).

Therefore, this work aims to evaluate the extraction of phenolic compounds from *J. regia* leaves by RSM using two extraction methods (ME and MAE), with ethanol:water mixtures as solvent, in order to obtain the following targets: 1) develop a process in a pre-industrial form for the extraction of 3-*O*-caffeoylquinic acid, quercetin 3-*O*-glucoside and quercetin *O*-pentoside from *J. regia* (walnut) leaves; and 2) optimize the primary variable conditions of the ME and MAE systems (time, temperature, ethanol-water proportion and solid-liquid ratio) and maximize the compounds extraction assisted by the statistical RSM technique, contributing to the understanding of the potential of *J. regia* leaves for industrial applications. In supplementary material (Fig. A1), a summary diagram containing the different steps carried out to obtain the optimal phenolic extract is presented.

2. Material and methods

2.1. Standards and reagents

HPLC-grade acetonitrile was from Fisher Scientific (Lisbon, Portugal). The phenolic compound standards (5-*O*-caffeoylquinic acid and quercetin 3-*O*-glucoside) were from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

2.2. Plant material

Juglans regia L. (walnut) dried leaves were purchased from Soria Natural, S.A., Spain. According to the distributor, the leaves were collected in Soria (Spain) in June 2014 and naturally dried in a room with controlled humidity. The samples were reduced to a fine powder (60–20 mesh) and stored in a desiccator protected from light for subsequent assays.

2.3. Extraction systems for walnut leaves

2.3.1. Maceration extraction

Solid-liquid maceration extractions (ME) of walnut leaves were carried out using a commercial Carousel (Carousel 12 Plus Reaction Station™, Radleys Tech) able to both stir and maintain the temperature within ± 0.5 °C, protected from light. The equipment is coupled to a refrigeration system, avoiding the loss of solvent. The powdered samples were extracted using different time (t, X_1), temperature (T, X_2) and ethanol proportion (S, X_3) conditions that ranged as defined by the RSM design (Table A1, supplementary material) maintaining the solid/liquid ratio (S/L, X_4) at 30 g/L. The S/L condition was further studied (5–140 g/L) after the optimization of X_1, X_2 and X_3 for each of the responses assessed. Samples were stirred at 600 rpm and the condensation/refrigeration system was kept at 15 °C. The solvent (ethanol, 0–100%, v/v) volume was fixed at 10 mL. After extraction, the mixture was centrifuged at 6000 rpm for 10 min at room temperature, the pellet was discarded and the supernatant was carefully collected for further analysis. The dry weight (dw) obtained from each solution was evaluated to determine the extraction yield (g extract/g sample).

2.3.2. Microwave-assisted extraction

The Microwave-assisted extraction (MAE) process was performed using a commercial microwave (Monowave 300, Anton Paar GmbH) in 30 mL closed vessels of high-precision glass. The solvent (ethanol 0–100%, v/v) volume was fixed at 10 mL. The powdered samples were extracted using the different conditions (t, T and S), as described by the RSM design (Table A1, supplementary material) maintaining S/L at 30 g/L. After, S/L was studied between 5 and 140 g/L for all the responses assessed. During processing, samples were stirred at 600 rpm using a magnetic stirring bar and irradiated at 850 W. In previous studies, other authors (Pinela et al., 2016) have shown that the microwave power has nearly no effect on the extraction process. Afterwards, the mixture in the extraction vessel was quickly cooled in the processing chamber until 55 °C. The mixture was centrifuged at 6000 rpm for 10 min at room temperature, the pellet was discarded and the supernatant was carefully collected for further analysis. The dry weight (dw) obtained from each solution was evaluated to determine the extraction yield (g extract/g sample).

Table 1
Bibliographic summary of the content of 3-*O*-caffeoylquinic acid (P1), quercetin 3-*O*-glucoside (P2) and quercetin *O*-pentoside (P3) from different source materials using different extraction techniques and conditions.

Technique	Plant Used for the Extraction		Content	Extraction Conditions					References
	Source material	Plant part		Solvent and proport.		Temp.	time	Others	
			mg/g dw	–	%	°C	min	–	
A: 3-*O*- Caffeoylquinic Acid Quantification (P1)									
ME	*Vaccinium floribundum* Kunth	Berries	0.015	EtOAC	100	RT	60	–	Prencipe et al. (2014)
	Ipomoea batatas L.	Leaf, stem & flower	0.13–0.83	MeOH:H_2O	80:20	RT	> 500	–	Jeng et al. (2015)
MAE	*Lonicera japonica* Thunb.	Flowers	0.89	EtOH:H_2O	50:50	40–70	3–12	–	Li et al. (2014)
UAE	*Solanum betaceum* Cav.	Skin, pulp & seeds	13.2–17.2	EtOH:H_2O	95:5	RT	30	–	Orqueda et al. (2017)
	Solanum betaceum Cav.	Yellow & purple cultivars	0.25–1.64	MeOH:H_2O	75:25	RT	60	–	Espin et al. (2016)
	Vaccinium corymbosum L.	Bluecrop, jersey & earliblue	0.06–0.61	MeOH:CH_2O_2	97:3	4–100	60	–	Zorenc et al. (2017)
HRE	*Lonicera japonica* Thunb.	Flowers	0.76	EtOH:H_2O	50:50	50	60	–	Li et al. (2014)
B: Quercetin Derivatives Quantification (P2 & P3)									
ME	*Salvia fruticosa* Mill.	Leaves	0.03	MeOH:H_2O	80:20	4	> 500	–	Sarrou et al. (2016)
	Aspalathus linearis (Burm. F.) R. Dahlgren	Leaves	0.49	H_2O	100	65–85	5–10	–	Santos et al. (2016)
	Vitis vinifera L.	Grape skin	< 1	CC:OA:H_2O	75:25	RT	> 500	–	Bubalo et al. (2016)
	Juglans regia L.	Leaves	7.41	MeOH	100	RT	120	–	Santos et al. (2013)
	V. floribundum	Berries	0.10 [a]	EtOAC	100	RT	60	–	Prencipe et al. (2014)
	Securigera securidaca L.	Flowers	33.40	EtOH:H_2O	90:10	4	–	–	Ibrahim et al. (2015)
	Rubus plicatus Weihe & Nees	Fruits	0.04	MeOH:CH_2O_2:H_2O	60:3:37	–	–	–	Kaume et al., (2012)
	Allium cepa L.	Red & gold onions	0.001–0.025 [a]	MeOH	100	RT	> 500	–	Bonaccorsi et al. (2008)
	Juglans regia L.	Leaves	7.64	H_2O	100	100 RT	10	–	Santos et al. (2013)
	Allium ascalonicum Hort.	French & Italian shallot	0.006–0.015 [a]	MeOH	100	RT	> 500	–	Bonaccorsi et al. (2008)
	Vitis vinifera L.	Grape berry skin White, red & black cultivars	0.006–0.080	MeOH:H_2O	80:20	RT	120	–	Ferreira et al. (2016)
MAE	*Vitis vinifera* L.	Grape skin	< 1	CC:OA:H_2O	75:25	65	50	–	Bubalo et al. (2016)
	Prunus laurocerasus L.	Leaves	1.75 [b]	MeOH	100	–	10–30	300–600 W	Karabegović et al. (2013)
UAE	*Potentilla thuringiaca* Bernh.	Herb	33.8 [b]	MeOH:H_2O	80:20	40	45	–	Tomczyk et al. (2012)
	Vitis vinifera L.	Grape skin	< 1	CC:OA:H_2O	75:25	65	50	35 kHz	Bubalo et al. (2016)
	Drymocallis rupestris (L.) Soják	Herb	26.6 [b]	MeOH:H_2O	80:20	40	45	–	Tomczyk et al. (2012)
	Myrica rubra Sieb. et Zucc.	Bayberry cultivars	2.13–8.76	EtOH:CH_2O_2:H_2O	95:1:4	RT	30	60 Hz, 30 W	Zhang et al. (2015)
	Dasiphora fruticosa L.	Herb	36.7	MeOH:H_2O	80:20	50	30	–	Tomczyk et al. (2012)
	Vaccinium corymbosum L.	Bluecrop, jersey & earliblue	0.10–0.22	MeOH:CH_2O_2	97:3	4–100	60	–	Zorenc et al. (2017)
	Vitis vinifera L.	Grape pomace	0.475–0.609	EtOH:H_2O	50:50	20	60	300W, 25 Hz	Drosou et al. (2015)
	Allium cepa L.	Red, yellow & chart. Onion	0.00–11.3	MeOH:CH_2O_2:H_2O	50:5:45	RT	35		Kwak et al. (2016)
	Potentilla nepalensis Hook	Leaves	27.1	MeOH:H_2O	80:20	50	30	–	Tomczyk et al. (2012)
	Allium cepa L.	Onion dry skin	0.09	MeOH:H_2O	80:20		1	–	Wiczkowski et al. (2014)
	Rosa canina L.	Hips	0.0078–0.0133	MeOH:CH_2O_2	97:3	–	60	–	Cunja et al. (2015)
SOXHLET	*Passiflora subpeltata* Ortega	Leaves	38.70 [c]	C_3H_6O	100	–	–	–	Shanmugam et al. (2016)
Pressurized Systems (SFE, PLE, ESE, SWE, SAE)	*Mangifera indica* L.	Leaves	0.4–2.3	CO_2:EtOH	80:20	55	180	10 MPa	Fernández-Ponce et al. (2012)
	Mangifera indica L.	Leaves	17.9–29.1 [b]	EtOH	100	60–100	–	4–20 MPa	Fernández-Ponce et al. (2015)
	Mangifera indica L.	Leaves	13.1–20.1 [b]	CO_2:EtOH:H_2O	50:25:25	60–100	–	12–20 MPa	Fernández-Ponce et al. (2015)
	Mangifera indica L.	Leaves	1.3–4.2	H_2O	100	100	180	4 MPa	Fernández-Ponce et al. (2012)
	Mangifera indica L.	By-products	5.95	C_3H_6O:H_2O	80:20	40	–	10 MPa	Meneses et al. (2015)

NOTATIONS: Supercritical Antisolvent Extraction (SAE); Enhanced Solvent Extraction (ESE); Pressurized Liquid Extraction (PLE); Supercritical Fluid Extraction (SFE); Subcritical Water Extraction (SWE); Heat Reflux Extraction (HRE); Microwave Assisted Extraction (MAE); Ultrasound Assisted Extraction (UAE); Maceration Extraction (ME); Methanol (MeOH); Ethyl acetate (EtOAC); Water (H_2O); Choline Chloride: Oxalic Acid (1:1) (CC:OA); Formic Acid (CH_2O_2); Ethanol (EtOH); Acetone (C_3H_6O); Room Temperature (RT); fresh weight ([a]); dry extract ([b]); mg/mL ([c]).

2.4. Quantification of the main compounds

2.4.1. Chromatographic analysis of the main phenolic compounds

After the ME and MAE processes, the extract solutions were filtered through 0.22 μm disposable LC filter disks. The samples were analysed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) with a quaternary pump and a photodiode array detector (PDA) coupled to an LC solution software data-processing station. Separation was achieved using a Waters Spherisorb S3 ODS-2C_{18}, (3 μm, 4.6 mm × 150 mm) column operating at 35 °C. The mobile phase was a mixture of formic acid in water 0.1% (A) and acetonitrile (B), and the established elution gradient was as follows: 15% B for 5 min, 15% B to 20% B over 5 min, 20–25% B over 10 min, 25–35% B over 10 min, 35–50% B for 10 min, and column re-equilibration (15 min), using a flow rate of 0.5 mL/min. Double online detection was carried with a diode array detector (DAD) operating at 280 and 370 nm as preferred wavelengths and the target phenolic compounds were identified according to their UV spectra and retention time (Barros et al., 2013). For the quantitative analysis, a baseline to valley integration with baseline projection mode was used to calculate peak areas and external standards were used for quantification. The results were expressed in mg per g of dry weight.

2.4.2. Determination of total phenolics and flavonols by UV–vis spectroscopy

The extract solutions obtained by the ME and MAE systems were filtered through *Whatman* n°4 filters. The extracts were diluted to obtain solutions with 79.8% v/v of ethanol. The extract solutions with different concentrations (0.25 mL) were mixed with ethanol:HCl (0.25 mL, HCl 0.1% v/v in the ethanol 95% solution) and HCl (4.55 mL, 2% v/v). The tubes were vortex mixed for 15 s and allowed to stand for 15 min at room temperature. The absorbance was then measured at 360 and 280 nm (total flavonols and total phenolics, respectively) in a Pharmaspec Shimadzu UV-1700 spectrophotometer. Quercetin 3-*O*-glucoside and chlorogenic acid were used to obtain the standard curves and results were expressed as mg of quercetin 3-*O*-glucoside or chlorogenic acid equivalents (QE or CAE, respectively) per g of dry plant.

2.5. Experimental design, model analysis and statistical evaluation

2.5.1. Experimental design

The study of the impact of all independent variables was carried using one-factor-at-a-time, to pick the most influents, and to determine the initial range of the processing variables. Through the analysis of this experimental 4. (data not shown), X_1 (t, min), X_2 (T, in °C) and X_3 (S, in %) were chosen as variables for the RSM design. Therefore, the combined effect of these three variables on the production of the three main phenolic compounds present in *J. regia* (maximizing responses individually or globally) was studied using central composite design with 5 levels of each factor. The experimental design is based on 20 independent combinations 6 of which are replicas at the central point of the experiment. The points were built around the centre point as a sphere. According to Box and Hunter (1957), the centre point is presumed to be close to the optimum position for the response, thus it is repeated to maximize the prediction. To minimize the unpredictable effects in the observed responses, experimental runs were random. The mathematical expressions used to calculate the design distribution, code and decode the tested variables can be found all detailed in the supplemental section (Table A1, supplementary material). Once the optimal conditions (X_1, X_2 and X_3) were optimized, the study was advanced furthermore with the study of the S/L condition (X_4, in g/L).

2.5.2. Mathematical model

The response surface models were fitted by means of least-squares calculation using the following second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

In this equation, Y represents the dependent variable (response variable) to be modelled, the independent variables are X_i and X_j, b_0 is the constant coefficient, b_i the coefficient of linear effect, b_{ij} the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and n is the number of variables. Chromatographic and spectrophotometric analysis in the dependent variable responses were used to quantify the main phenolic compounds.

2.5.3. Procedure to optimize the variables to a maximum response

A simplex method was used to maximize the model process by solving non-linear problems to optimize the quercetin 3-*O*-glucoside, quercetin *O*-pentoside, 3-*O*-caffeoylquinic acid, yield, total phenolics and flavonols extraction (Heleno et al., 2016; Pinela et al., 2016). To avoid unnatural conditions of the variables certain limitations were imposed (*i.e.*, times lower than 0).

2.6. Fitting procedures and statistical analysis

The experimental results statistical analysis and fitting was built according to the equations for the responses obtained using a Microsoft Excel spreadsheet in three phases:

- Coefficients measurement was achieved using the nonlinear least-square (quasi-Newton) method provided by the macro Solver in Microsoft Excel (Kemmer and Keller, 2010), by minimization of the sum of quadratic differences between observed and model-predicted values.
- Coefficients significance was obtained *via* 'SolverAid' (Prikler, 2009) to determine the parametric confidence intervals. The terms that were not statistically significant (p-value > 0.05) were dropped to simplify the model.
- Model reliability was confirmed by applying the following standards: a) the Fisher F-test ($\alpha = 0.05$) was used to determine the consistency of the constructed models to describe the obtained data (Shi and Tsai, 2002); b) the 'SolverStat' macro was used to make assessment of parameter and model prediction uncertainties (Comuzzi et al., 2003); c) R^2 was determined to explain the proportion variability of the dependent variable obtained by the model.

3. Results and discussion

3.1. Response criteria for the RSM analysis and statistical verification

The phenolic profile of *J. regia* leaves extracts is represented in Fig. A2 (supplementary material). The main compounds present in the matrix were one phenolic acid, 3-*O*-caffeoylquinic acid (P1) and two flavonols, quercetin 3-*O*-glucoside (P2) and quercetin *O*-pentoside (P3). This profile agrees with the previous one reported by Santos et al. (Santos et al., 2013). These compounds were identified by comparison of their UV spectra and retention times with those obtained in previous findings, as well as with the commercial standards. Both quantification results (HPLC-PDA and UV–vis) are represented in Table 2 for the different runs of the RSM design.

Regarding HPLC-PDA results, the levels of the two flavonols ranged from 3.9 to 13.7 mg/g dw for P2 with the ME process (runs 14 and 15) and 6.4–14.7 mg/g dw using the MAE system (runs 13 and 7) and for P3 between 3.7–13.1 mg/g dw by employing the ME (runs 14 and 15) and 5.4–13.6 mg/g dw through the MAE (runs 12 and 7) techniques. The amounts of 3-*O*-caffeoylquinic acid (P1) ranged from 0.0 (not detected, under the LOQ) to 5.8 mg/g dw using the ME (runs 7 and 3) system and 1.2–6.3 mg/g dw applying the MAE process (runs 14 and 2).

The amounts of total phenolics and flavonols were also determined

Table 2
Results of the response surface experimental plan for the ME and MAE optimization process of independent variables of time (*t*), temperature (*T*), and solvent proportion (*S*). Response criteria comprise the following: 1) % yield of extraction; 2) HPLC quantification of 3-*O*-caffeoylquinic acid content (P1), quercetin 3-*O*-glucoside content (P2), quercetin *O*-pentoside content (P3) and total HPLC content (P1 + P2 + P3); and 3) spectrophotometric quantification of total phenolics (PHE) and flavonols (FLAV).

	Experimental Design									Maceration Responses							Microwave Responses						
	Coded values			*Maceration conditions*			*Microwave conditions*			*Residue*	*HPLC Content*				*Others*		*Residue*	*HPLC Content*				*Others*	
	X_1	X_2	X_3	X_1: *t* min	X_2: *T* °C	X_3: *S* %	X_1: *t* min	X_2: *T* w	X_3: *S* %	*Yield* %	*P1* mg/g dw	*P2* mg/g dw	*P3* mg/g dw	*Total* mg/g dw	*PHE* mg/g dw	*FLAV* mg/g dw	*Yield* %	*P1* mg/g dw	*P2* mg/g dw	*P3* mg/g dw	*Total* mg/g dw	*PHE* mg/g dw	*FLAV* mg/g dw
1	−1	−1	−1	54.3	42.2	20.3	11.5	85	20.3	22.53	5.0	6.7	7.2	19.0	72.3	32.6	24.86	5.9	8.5	9.0	23.5	60.2	26.4
2	1	−1	−1	125.7	42.2	20.3	36.5	85	20.3	23.66	5.6	6.8	7.2	19.7	75.0	33.6	25.65	6.3	7.6	7.1	21.0	66.8	29.4
3	−1	1	−1	54.3	77.9	20.3	11.5	155	20.3	25.29	5.8	7.7	8.1	21.6	90.6	40.0	38.60	6.0	8.2	8.5	22.6	125.4	50.4
4	1	1	−1	125.7	77.9	20.3	36.5	155	20.3	25.45	5.3	6.6	6.5	18.5	90.4	39.4	37.90	5.9	7.1	6.2	19.3	126.9	48.2
5	−1	−1	1	54.3	42.2	79.7	11.5	85	79.7	19.13	1.3	8.1	9.5	18.9	86.9	43.2	24.60	2.4	14.0	13.0	29.4	105.4	50.4
6	1	−1	1	125.7	42.2	79.7	36.5	85	79.7	20.84	1.3	7.6	9.3	18.2	95.7	47.4	25.41	2.5	14.3	13.4	30.2	114.6	54.0
7	−1	1	1	54.3	77.9	79.7	11.5	155	79.7	23.67	0.0	8.2	10.4	18.6	121.4	58.1	30.77	3.2	14.7	13.6	31.5	167.7	70.3
8	1	1	1	125.7	77.9	79.7	36.5	155	79.7	24.18	1.3	8.3	10.0	19.5	96.6	44.4	32.46	3.3	13.8	12.7	29.8	179.8	74.2
9	−1.68	0	0	30	60	50	3	120	50	25.85	2.1	12.6	11.9	26.7	98.9	44.3	28.80	3.8	14.3	12.8	30.9	121.5	51.2
10	1.68	0	0	125.7	60	50	45	120	50	26.77	2.2	13.6	12.6	28.5	110.3	47.5	31.99	3.6	14.1	12.5	30.2	128.7	54.4
11	0	−1.68	0	90	30	50	24	60	50	23.77	1.9	12.7	11.7	26.4	77.1	36.2	26.26	3.7	14.4	12.4	30.5	100.3	45.7
12	0	1.68	0	90	90	50	24	180	50	29.15	2.4	13.6	12.8	28.7	129.3	54.8	44.26	4.4	9.4	5.4	19.2	194.9	65.8
13	0	0	−1.68	90	60	0	24	120	0	19.71	0.6	5.2	4.8	10.6	58.6	23.8	27.32	6.4	6.4	6.1	18.9	74.4	30.1
14	0	0	1.68	90	60	100	24	120	100	11.11	0.3	3.9	3.7	8.0	49.4	25.6	17.65	1.2	11.8	13.3	26.3	65.3	34.3
15	0	0	0	90	60	50	24	120	50	25.98	2.4	13.7	13.1	29.2	113.2	50.2	30.28	3.6	14.1	12.9	30.7	129.4	53.6
16	0	0	0	90	60	50	24	120	50	25.65	2.2	13.5	12.7	28.4	111.8	48.9	31.47	3.5	14.1	12.1	29.6	119.0	51.0
17	0	0	0	90	60	50	24	120	50	26.25	2.1	12.9	12.1	27.1	106.6	46.4	34.41	3.5	14.2	13.1	30.9	124.2	52.3
18	0	0	0	90	60	50	24	120	50	25.98	2.3	13.4	12.9	28.6	104.0	45.2	32.81	3.5	14.4	13.5	31.4	124.0	53.4
19	0	0	0	90	60	50	24	120	50	25.58	2.2	13.1	12.6	27.9	107.2	46.4	30.76	3.5	14.1	13.2	30.8	123.7	52.9
20	0	0	0	90	60	50	24	120	50	26.57	2.4	13.5	13.0	29.0	108.9	47.5	31.29	3.4	14.4	13.4	31.3	127.2	54.2

Table 3
Estimated coefficient values obtained from the Box polynomial model, parametric intervals and numerical statistical criteria for each parametric response criteria of the extractions systems tested (ME and MAE). Response criteria comprise the following: 1) % yield of extraction; 2) HPLC quantification of 3-*O*-caffeoylquinic acid content (P1), quercetin 3-*O*-glucoside content (P2), quercetin *O*-pentoside content (P3) and total HPLC content (P1 + P2 + P3); and 3) spectrophotometric quantification of total phenolics (PHE) and flavonols (FLAV).

Parameters		Residue	HPLC Content				Others	
		Yield	*P1*	*P2*	*P3*	*Total*	*PHE*	*FLAV*
Maceration Extraction								
Intercept	b_0	26.23 ± 0.38	2.25 ± 0.16	13.26 ± 0.73	12.68 ± 0.47	28.28 ± 0.83	109.2 ± 3.64	48.16 ± 1.75
Linear effect	b_1	0.55 ± 0.29	ns	0.17 ± 0.12	0.12 ± 0.02	0.30 ± 0.11	6.29 ± 1.94	2.17 ± 1.07
	b_2	1.50 ± 0.38	ns	0.20 ± 0.12	0.24 ± 0.02	0.21 ± 0.11	11.49 ± 3.01	3.60 ± 1.39
	b_3	−1.95 ± 0.36	−2.43 ± 0.12	0.28 ± 0.12	0.54 ± 0.02	ns	4.15 ± 3.01	2.98 ± 1.31
Quadratic effect	b_{11}	ns	ns	−0.15 ± 0.12	−0.10 ± 0.02	−0.19 ± 0.11	−3.50 ± 1.36	−1.37 ± 0.72
	b_{22}	ns	0.17 ± 0.11	−0.39 ± 0.12	−0.23 ± 0.02	−0.25 ± 0.11	ns	ns
	b_{33}	−3.66 ± 0.31	0.91 ± 0.10	−3.39 ± 0.12	−3.18 ± 0.02	−7.15 ± 0.11	−17.88 ± 2.90	−7.03 ± 1.17
Interactive effect	b_{12}	ns	−0.28 ± 0.16	−0.20 ± 0.12	−0.32 ± 0.02	−0.22 ± 0.11	−4.55 ± 3.93	−1.85 ± 2.09
	b_{13}	ns	ns	ns	ns	0.37 ± 0.11	−2.30 ± 2.20	ns
	b_{23}	ns	0.20 ± 0.17	ns	0.052 ± 0.02	ns	ns	ns
Statistics (R^2)		0.9240	0.9662	0.8706	0.9211	0.9488	0.9021	0.8965
Microwave Assisted Extraction								
Intercept	b_0	31.81 ± 0.68	3.50 ± 0.18	14.24 ± 0.60	12.95 ± 0.50	30.74 ± 1.12	125.3 ± 6.07	53.41 ± 2.73
Linear effect	b_1	0.58 ± 0.45	ns	−0.21 ± 0.19	−0.37 ± 0.22	−0.57 ± 0.54	3.04 ± 2.71	1.05 ± 0.97
	b_2	5.09 ± 0.45	0.18 ± 0.12	−0.65 ± 0.40	−0.99 ± 0.39	−1.47 ± 0.74	30.16 ± 4.74	8.54 ± 2.14
	b_3	−2.20 ± 0.45	−1.58 ± 0.12	2.52 ± 0.40	2.49 ± 0.39	3.44 ± 0.74	12.67 ± 4.74	7.43 ± 2.14
Quadratic effect	b_{11}	−0.34 ± 0.33	0.19 ± 0.11	−0.13 ± 0.12	ns	ns	ns	ns
	b_{22}	1.38 ± 0.44	0.30 ± 0.11	−0.94 ± 0.38	−1.41 ± 0.38	−2.05 ± 0.72	9.05 ± 4.59	1.74 ± 1.22
	b_{33}	−3.14 ± 0.44	0.23 ± 0.11	−1.92 ± 0.38	−1.14 ± 0.38	−2.84 ± 0.72	−18.45 ± 4.59	−6.57 ± 2.07
Interactive effect	b_{12}	ns	ns	ns	ns	ns	ns	ns
	b_{13}	ns	ns	ns	ns	ns	ns	ns
	b_{23}	−1.60 ± 0.59	0.26 ± 0.15	ns	ns	ns	ns	ns
Statistics (R^2)		0.9791	0.9614	0.9011	0.8903	0.9224	0.9588	0.8874

using UV–vis measurements. Observing the total phenolic content, the levels ranged from 49.4 to 129.3 mg CAE/g dw (runs 14 and 12) and 23.8–58.1 mg QE/g dw (runs 13 and 7) using the ME system and 60.2–194.9 mg CAE/g dw (runs 1 and 12) and 26.4–74.2 mg QE/g dw (runs 1 and 8) applying the MAE process.

Concerning the extraction yields, both techniques are consistent: the highest yield was obtained in run 12 (29.15% using ME and 44.26% with MAE) and the lowest in run 14 (11.11% using ME system and 17.65% with MAE).

Overall, applying the RSM conditions (Table 2) to the MAE technique not only lead to higher amounts of P1, P2, P3, total phenolics and flavonols but also higher extraction yields. Accordingly, the sum of the three main compounds (P1 + P2 + P3) using the HPLC-PDA measurements was also superior for the MAE process (run 7: 31.5 mg/g dw) than the ME system (run 15: 29.2 mg/g dw). The quantification results obtained from the chromatographic and spectrophotometric methodologies were used as response criteria to optimize the ME and MAE conditions by RSM. The obtained parametric fitting values, confidence intervals and statistical information are presented in Table 3. All coefficients showed significant parametric intervals at the 95% confidence level ($\alpha = 0.05$) and the correlation coefficients were always higher than 0.87.

3.2. Theoretical response surface models

It is essential to evaluate the precision of the RSM mathematical model on the ideal prediction of variances. Fitting the models to the selected responses is the key to elucidate this prediction; therefore, the models for each individual response were constructed fitting the second-order polynomial model of Eq. (1) to the experimental values in Table 3 using nonlinear least-squares estimations. Therefore, the resulting models for each assessed extraction technique are the following, for the ME system:

$$Y_{yield}^{ME} = 26.2 + 0.55t + 1.5T - 2.0S - 3.7S^2 \quad (2)$$

$$Y_{P1}^{ME} = 2.3 - 2.4S + 0.17T^2 + 0.91S^2 - 0.28tT + 0.20TS \quad (3)$$

$$Y_{P2}^{ME} = 13.3 + 0.17t + 0.20T + 0.28S - 0.15t^2 - 0.39T^2 - 3.4S^2 - 0.20tT \quad (4)$$

$$Y_{P3}^{ME} = 12.7 + 0.12t + 0.24T + 0.54S - 0.1t^2 - 0.23T^2 - 3.18S^2 - 0.32tT + 0.05TS \quad (5)$$

$$Y_{P1+P2+P3}^{ME} = 28.3 + 0.30t + 0.21T - 0.19t^2 - 0.25T^2 - 7.1S^2 - 0.22tT + 0.37tS \quad (6)$$

$$Y_{PHE}^{ME} = 109.2 + 6.3t + 11.5T + 4.2S - 3.5t^2 - 17.9S^2 - 4.6tT - 2.3tS \quad (7)$$

$$Y_{FLAV}^{ME} = 48.2 + 2.2t + 3.6T + 3.0S - 1.4t^2 - 7.0S^2 - 1.9tT \quad (8)$$

And for the MAE process:

$$Y_{yield}^{MAE} = 31.8 + 0.58t + 5.1T - 2.2S - 0.34t^2 + 1.4T^2 - 3.1S^2 - 1.6TS \quad (9)$$

$$Y_{P1}^{MAE} = 3.5 + 0.18T - 2.6S + 0.19t^2 + 0.30T^2 + 0.23S^2 + 0.26TS \quad (10)$$

$$Y_{P2}^{MAE} = 14.2 - 0.21t - 0.65T + 2.5S - 0.13t^2 - 0.94T^2 - 1.9S^2 \quad (11)$$

$$Y_{P3}^{MAE} = 13.0 - 0.37t - 0.99T + 2.5S - 1.4T^2 - 1.1S^2 \quad (12)$$

$$Y_{P1+P2+P3}^{MAE} = 30.7 - 0.57t - 1.5T + 3.4S - 2.1T^2 - 2.8S^2 \quad (13)$$

$$Y_{PHE}^{MAE} = 125.3 + 3.0t + 30.2T + 12.7S + 9.1T^2 - 18.5S^2 \quad (14)$$

$$Y_{FLAV}^{MAE} = 53.4 + 1.1t + 8.5T + 7.4S + 1.7T^2 - 6.6S^2 \quad (15)$$

These equations translate the response patterns for individual measurement of phenolic compounds and Table 3 shows the complexity of possible sceneries. Not all the parameters of Eq. (1) were used for building the model since some coefficients were non-significant.

Regarding the linear effect on the ME process, the effect of t and T were significant in a positive mode in all cases except for 3-*O*-caffeoylquinic acid (no significant effects were found). The variable S played an important and significant role for the yield and 3-*O*-caffeoylquinic acid in a negative way, but positive for the quercetin

derivatives, total phenolics and flavonols. However, no significant effect was found for the total HPLC content. Observing the quadratic effect, *t* exerts a negative significant role to quercetin derivatives, total HPLC content, total phenolics and flavonols, *T* also plays a negative significant effect for the quercetin derivatives and total HPLC content, but positive for the 3-*O*-caffeoylquinic acid. The variable *S* also played a positive role for 3-*O*-caffeoylquinic acid and negative to the remaining ones. On the other hand, the interactive effect just had a significant effect in some cases: the interactions between *t* & *T* caused a significant negative effect in all responses except for the yield where no significant interactions were found. The interaction between *t* & *S* were only significant for the total HPLC content (positive) and total phenolics (negative) responses. The variables interactions *T* & *S* caused significant positive effects on 3-*O*-caffeoylquinic acid and quercetin *O*-pentoside.

Regarding the MAE process, the linear effect had the most important and significant role for all the variables with exception of *t* on the 3-*O*-caffeoylquinic acid content (no significant effect was found). The effect of *t* was significant in a positive mode for the yield, total phenolic content and flavonols content and negative for the remaining HPLC measurements. The variable *T* showed a significant positive effect for yield, 3-*O*-caffeoylquinic acid, total phenolics and flavonols and a negative effect on quercetins derivatives and total HPLC content. The variable *S* played an important and significant role for the yield and 3-*O*-caffeoylquinic acid in a negative mode, however, positive to quercetin derivatives, total HPLC, total phenolics and flavonols. Regarding the quadratic effect, *t* exerts a negative significant role in yield and quercetin 3-*O*-glucoside, but positive on 3-*O*-caffeoylquinic acid response, *T* plays an important and significant role in all responses (positive: yield, 3-*O*-caffeoylquinic acid, total phenolics and flavonols; negative: quercetin derivatives and total HPLC content), the variable *S* also exerts significant influence in all parameters of this study: positive to 3-*O*-caffeoylquinic acid and negative to the remaining ones. Observing the interactive effect, the interaction between *T* & *S* only presented a significant effect on the response of yield and 3-*O*-caffeoylquinic acid (negative and positive, respectively).

3.3. Effect of extraction variables on the target responses

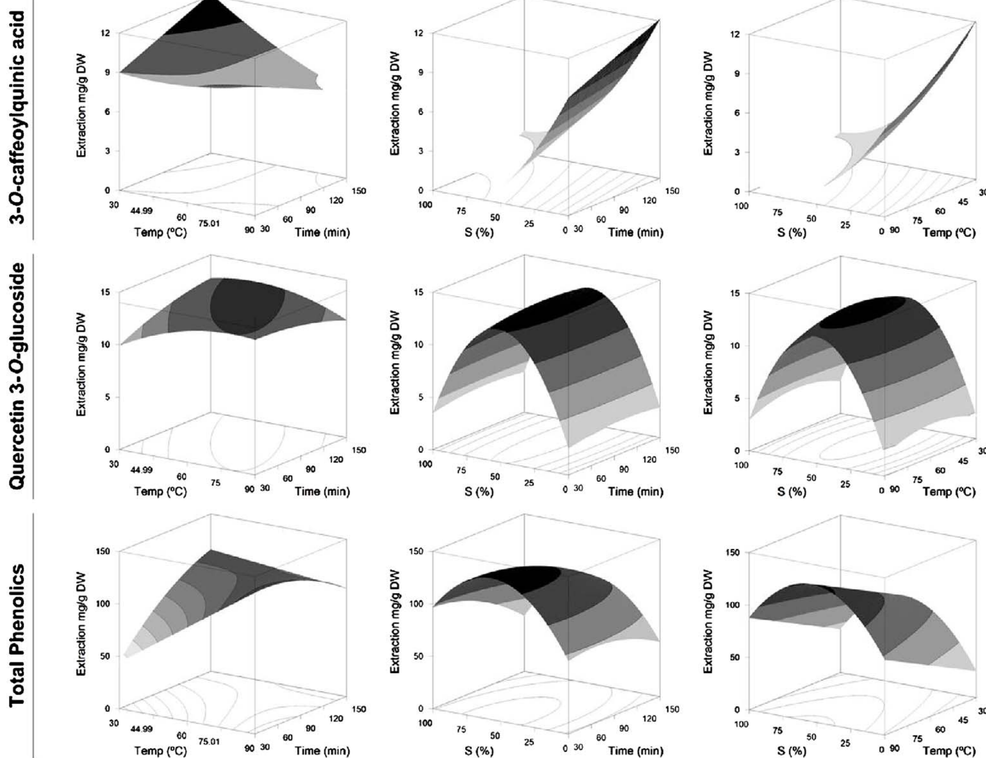
The patterns of extraction can also be depicted by graphical representation. Fig. 1 represents a 3D graphical analysis of the results in mg/g dw of 3-*O*-caffeoylquinic acid and quercetin 3-*O*-glucoside by HPLC quantification and total phenolics by spectrophotometric determination obtained for both extraction systems. Fig. 2 represents the results in mg/g dw of total HPLC and yield. They are divided in two parts (columns) for each tested technique. Each column is divided into two subsections (A and B). Subsection A illustrates a 3D response surface plots predicted with their respective second order polynomial equation described by Eqs. (6), (2), (13) and (9). In this subsection, a binary representation is shown, in which the excluded variable is positioned at its optimal experimental domain. Subsection B shows the capability to forecast the achieved results and the residual distribution as a function of the individual considered variables.

Observing all the plots in Fig. 1 it is possible to verify that the amount of extracted material increases to an optimum value and, in most cases, decreases as a function of each assessed independent variable. Similar behaviour can be found in Figs. 2 and A3 (supplementary material). Consequently, in almost all combinations the optimum value in single points can be found, which allows computing the conditions that lead to an absolute maximum. Fig. 3 simplifies the interpretation of the influence of the independent variables in a bi-dimensional representation of the optimal conditions of the RSM variables.

Part A of Fig. 3 shows a two-dimension representation of the influence of the independent variables relative to each extraction method. The operating conditions that maximize the extraction conditions are represented with dots (⊙), these singular conditions are represented in Table 4 to clarify not only the optimal independent variables conditions but also the optimal values predicted. The experimental results are also presented in this table.

Regarding the particular case of the ME system (Fig. 3), the intense curvature of the solvent observed on the total HPLC contents dictates its action: when raising the percentage of ethanol until 50% the extraction content also increases (~28 mg/g dw – represented by ⊙), followed afterwards by a decrease of this content. Higher amounts 3-*O*-caffeoylquinic acid are obtained when using 0% of ethanol (100% water) and quercetin derivatives with 50% of solvent. As quercetin derivatives are present in higher amounts comparatively to 3-*O*-caffeoylquinic acid, the optimal for total HPLC content is obtained in similar conditions of quercetin derivatives. Therefore, as can be seen in Table 4, the maximum global optimal variable conditions for the total compounds reached by the ME system were 115.6 min (X_1), 61.3 °C (X_2) and 50.4% of ethanol (X_3), predicting 28.3 mg/g dw (28.8 mg/g dw experimental). Since the total HPLC is the result of the sum of the obtained responses regarding the 3-*O*-caffeoylquinic acid (10.7 mg/g dw predicted, 11.0 mg/g dw experimental), quercetin 3-*O*-glucoside (13.2 mg/g predicted, 13.8 mg/g dw experimental) and quercetin *O*-pentoside (12.7 mg/g dw predicted, 11.7 mg/g dw experimental) amounts, the optimal variable conditions should be around the ones which are in higher quantities individually. Due to structural similarities, quercetin derivatives present close to optimal conditions of extraction with this technique (quercetin 3-*O*-glucoside: 112.5 min, 62.93 °C and 50% of ethanol; quercetin *O*-pentoside: 112.5 min, 62.9 °C and 50.6% of ethanol), thus they exert a strong influence on the final conditions to the optimal variable conditions on the total HPLC content. However, using the MAE process, factors beyond structural similarities are essential for the explanation of the obtained results. In this technique, the energy provided by microwaves is applied facilitating the partition of molecules present in the matrix into the solvent (Dai and Mumper, 2010). Consequently, the behaviour of the independent variables are not as obvious as those observed in the ME system and unexpected results were obtained when applying the MAE process. In fact, for quercetin derivatives extraction proximal conditions of *T* and *S* were observed (quercetin 3-*O*-glucoside: 107.81 °C and 69.4% of ethanol; quercetin *O*-pentoside: 107.7 °C and 82.5% of ethanol) as equal optimum responses (quercetin 3-*O*-glucoside: 15.2 and 14.2 mg/g dw, predicted and experimental, respectively; quercetin *O*-pentoside: 15.1 and 14.0 mg/g dw, predicted and experimental, respectively). Nevertheless, a discrepancy was found in the time of extraction (quercetin 3-*O*-glucoside: 14.1 min; quercetin *O*-pentoside: 3.0 min). At first, it can be seen that times are shorter for the MAE than the ME techniques (as expected), and second, the kinetics of extraction of each compound (although they are structurally quite similar) are singular: quercetin *O*-pentoside shows a faster optimum value than quercetin 3-*O*-glucoside. As observed on the MAE process, optimal response on 3-*O*-caffeoylquinic acid was obtained with 0% of ethanol, and increasing the temperature up to 61.1 °C (ME: 29.9 °C) decreases the time of extraction until 3.0 min, compared to 150.0 min for the ME system optimal conditions (predicted: 10.7 mg/g dw; experimental: 11.0 mg/g dw). Thus, comparing the predicted and experimental values obtained through HPLC maximized conditions (individual and sum of main compounds), it can be concluded that polynomial equations are well adjusted. Concerning the individual optimal variable conditions for the UV–vis determinations, the maximum values for each extraction technique were achieved in similar conditions. In fact, for the ME system, the total phenolics (81.1 min, 90.0 °C, 53.9% of ethanol) and total flavonols (77.7 min, 90.0 °C, 56.3% of ethanol) conditions are close. The same was observed in the MAE process for the total phenolics (45.0 min, 180 °C, 60.2% of ethanol) and total flavonols (45.0 min, 180.0 °C, 66.8% of ethanol). The predicted and experimental values obtained by UV–vis measurements were also satisfactory. Regarding the total phenolics, the predicted response using the ME system was 129.1 mg CAE/g dw, but the experimental result was 124.0 mg CAE/g dw, and for the MAE process the predicted value was 209.1 mg CAE/g dw, and the

MACERATION EXTRACTION



MICROWAVE ASSISTED EXTRACTION

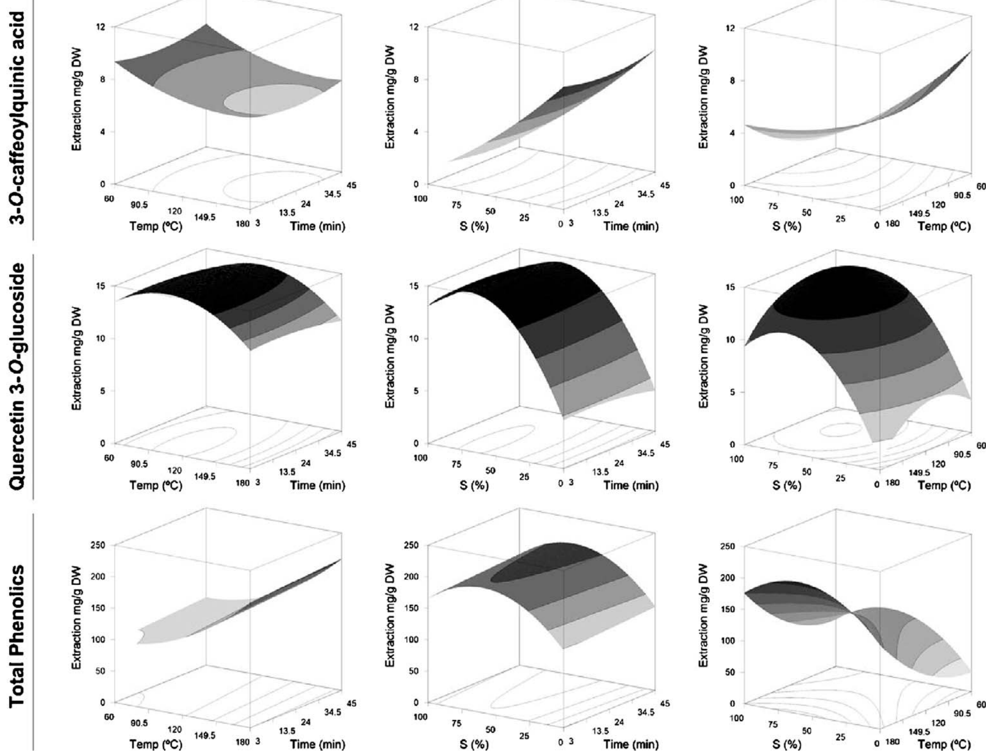


Fig. 1. Shows the joint graphical 3D analysis as a function of each the variables involved for ME and MAE systems for the HPLC quantification of 3-*O*-caffeoylquinic acid content (P1) and quercetin 3-*O*-glucoside content (P2) and for the spectrophotometric determination of the total phenolics (PHE). The other response determinations of quercetin *O*-pentoside content (P3) and total flavonols (FLAV) were excluded and presented in Fig. A3 (supplemental material) due to their similarity with quercetin 3-*O*-glucoside content (P2) and total phenolics (PHE), respectively. Each of the net surfaces represents the theoretical three-dimensional response surface predicted with the second order polynomial of Eqs. (3), (4) and (7) in ME system and (10), (11) and (14) for MAE process. The statistical design and results are described in Table 2. Estimated parametric values are shown in Table 3. The binary actions between variables are presented when the excluded variable is positioned at the optimum of the experimental domain (Table 4).

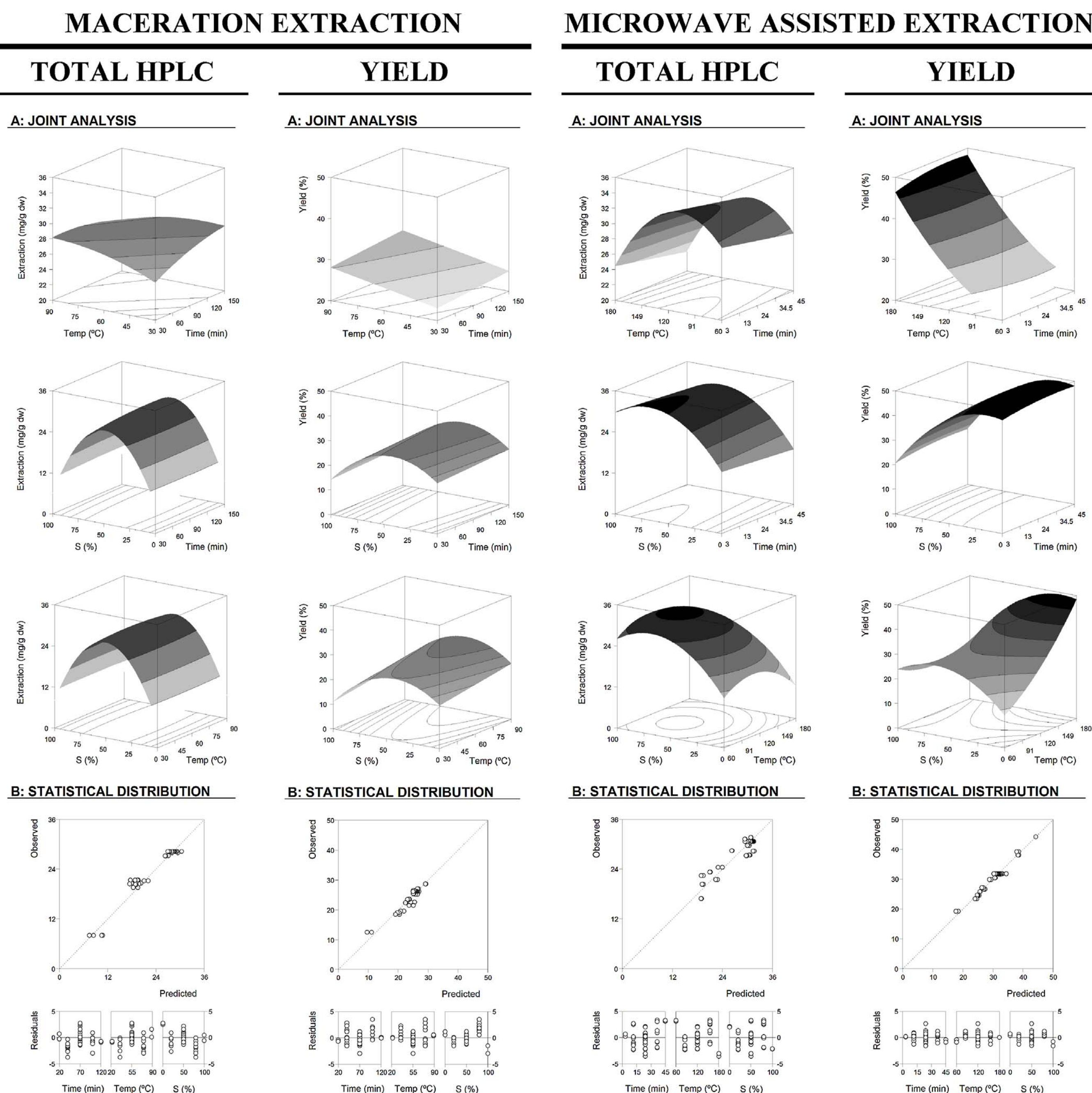


Fig. 2. Shows the graphical results in terms of the response surfaces of the total compounds content determined by HPLC (P1 + P2 + P3) and extraction yield from the developed equations for the ME and MAE systems optimization. Part A: Shows the joint graphical 3D analysis as a function of each of the variables involved. Each of the net surfaces represents the theoretical three-dimensional response surface predicted with the second order polynomial of Eqs. (6), (2), (13) and (9), respectively. The statistical design and results are described in Table 2. Estimated parametric values are shown in Table 3. The binary actions between variables are presented when the excluded variable is positioned at the optimum of the experimental domain (Table 4). Part B: To illustrate the goodness of fit, two basic graphical statistic criteria are used. The first one, the ability to simulate the changes of the response between the predicted and observed data; and the second one, the residual distribution as a function of each of the variables. Note all the differences in the axes scales.

experimental 194.9 mg CAE/g dw. The same findings were observed in total flavonols predicted and experimental results, applying the ME technique (54.7 and 53.3 mg QE/g dw, respectively) and the MAE process (66.5 and 66.0 mg QE/g dw, respectively). The particular optimal variable conditions in yield residue extraction were different for the ME and MAE systems. The optimal response for the ME system (29.94% predicted and 29.3% experimental) were obtained at 150.0 min, 90.0 °C and 42.1% of ethanol. In contrast, the optimum values obtained for the MAE process (46.4 and 44.3%, predicted and experimental) were reached at 34.6 min, 180.0 °C and 60.2% of ethanol. Thus, the MAE process leads to higher extraction yields in a shorter time. This profile of extraction was also verified for the remaining measured parameters. In fact, in all cases, the MAE process leads to higher extraction yields (mg/g dw, mg CAE/g dw or mg QE/g dw) with shorter extraction times.

Due to the accurate HPLC results, the studies on S/L were performed in optimal conditions predicted by the polynomial models obtained for each extraction technique using the total HPLC content as the response factor (Table 4). Preliminary results (data not shown) indicate that the experimental limit value of concentration was proximal to 140 g/L due to the conditioned experimental stirring for higher S/L values. Therefore, both processes of extraction were designed to verify the S/L

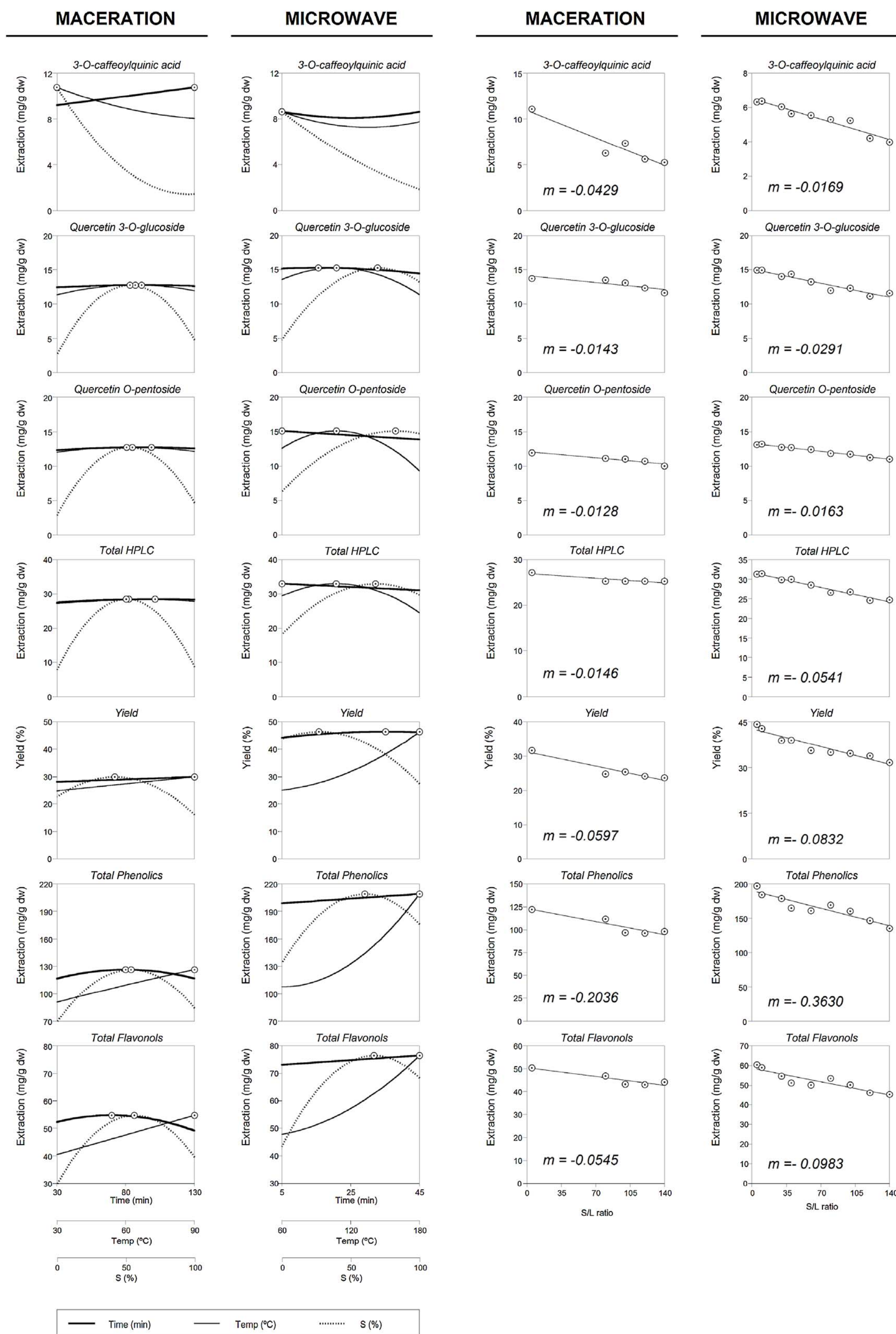


Fig. 3. Final summary of the effects of all variables assessed for ME and MAE systems. *Part A*: Shows the individual 2D responses of all studied responses as a function of all the variables assessed. The variables in each of the 2D graphs were positioned at the optimal values of the others (Table 4). The dots (⊙) presented alongside each line highlight the location of the optimum value. Lines and dots are generated by the theoretical second order polynomial models of Eqs. (2)–(7) for ME and (9)–(15) for MAE. *Part B*: Shows the dose response of *S/L ratio* at the optimal values of the other three variables (Table 4). Points (○) represent the obtained experimental results, meanwhile the line shows the predicted pattern by simple linear relation. The limit value (~140 g/L) shows the maximum achievable experimental concentration until the sample cannot be physically stirred at laboratory scale.

Table 4
Operating optimal conditions of the variables involved that maximize the response values for RSM using a CCCD for each of the extracting techniques assessed (ME and MAE), for the three individual response value formats of each compound and for all the compounds. Response criteria comprise the following: 1) % yield of extraction; 2) HPLC quantification of 3-*O*-caffeoylquinic acid content (P1), quercetin 3-*O*-glucoside content (P2), quercetin *O*-pentoside content (P3) and total HPLC content (P1 + P2 + P3); and 3) spectrophotometric quantification of total phenolics (PHE) and flavonols (FLAV). The optimum values underlined are the results found experimentally, meanwhile the others are those found by the mathematical model.

Criteria	*Optimal Variable Conditions*			*Optimum Response*	
	X_1: t (min)	*X_2: T (°C)*	*X_3: S (%)*	*(mg/g dw)*	
Individual optimal variable conditions for 3-O-caffeoylquinic acid:					
Maceration	150.0	29.9	0.0	10.7 ± 1.45	<u>11.0</u>
Microwave	3.0	61.1	0.0	8.6 ± 0.98	<u>6.4</u>
Individual optimal variable conditions for the quercetin 3-O-glucoside:					
Maceration	112.5	62.93	50.6	13.2 ± 1.37	<u>13.8</u>
Microwave	14.1	107.81	69.4	15.2 ± 1.11	<u>14.2</u>
Individual optimal variable conditions for the quercetin O-pentoside:					
Maceration	112.5	62.9	50.6	12.7 ± 1.23	<u>11.7</u>
Microwave	3.0	107.7	82.5	15.1 ± 0.95	<u>14.0</u>
Global optimal variable conditions for the total HPLC compounds:					
Maceration	115.6	61.3	50.4	28.3 ± 2.43	<u>28.8</u>
Microwave	3.0	107.5	67.9	32.9 ± 3.16	<u>29.3</u>
Individual optimal variable conditions for the yield residue extraction:					
Maceration	150.0	90.0	42.1	29.9 ± 1.22	<u>29.3</u>
Microwave	34.6	180.0	26.8	46.4 ± 4.15	<u>44.3</u>
Individual optimal variable conditions for the total phenolics:					
Maceration	81.1	90.0	53.9	129.1 ± 23.46	<u>124.0</u>
Microwave	45.0	180.0	60.2	209.1 ± 16.21	<u>194.9</u>
Individual optimal variable conditions for the total flavonols:					
Maceration	77.7	90.0	56.3	54.7 ± 2.98	<u>53.3</u>
Microwave	45.0	180.0	66.8	66.5 ± 6.56	<u>66.0</u>

behaviour between 5 g/L until 140 g/L. The dose response of S/L ratio is presented on Part B of Fig. 3. The responses obtained through the ME and the MAE systems are consistent: the S/L ratio can be described by a simple linear relationship. All experimental points are distributed around the equation with only one independent variable, consequently the dose response is explained by the slope (m) of the linear relation. The S/L increase leads to a decrease in the extraction ability of the solvent; consequently, the extraction responses reach a maximum value at 5 g/L and a minimum at 140 g/L and the respective losses are justified by the m value. However, the decreases observed are slight (less than −0.4). The m values in 3-*O*-caffeoylquinic acid for the ME and MAE techniques were −0.0429 and −0.0169, meaning that for the ME system, the increase of 1 g/L on S/L implies the loss of 0.0429 mg/g dw and for the MAE process 0.0169 mg/g dw. Although the ME system leads to higher losses of 3-*O*-caffeoylquinic acid with the optimal proposed conditions, for the final S/L, it remains better than the MAE process. In fact, the ME system is more efficient to extract this phenolic acid (ME: ~11 to 5 mg/g dw; MAE: ~6 to 4 mg/g dw). However, the same tendency is not verified in the remaining responses. In fact, the parameter m assumes higher absolute values in the MAE process compared to those found in the ME system (quercetin 3-*O*-glucoside: −0.0291 and −0.0143; quercetin *O*-pentoside: −0.0163 and −0.0128; total HPLC: −0.0541 and −0.0146; yield: −0.0832 and −0.0597; total phenolics: −0.3630 and −0.2036; total flavonols: −0.0983 and −0.0545). The higher loss (expressed on mg/g) induced by the increase of S/L was verified for the total phenolics amounts obtained by the ME system ($m = -0.3630$). However, the amounts reached at 140 g/L using the MAE process (~ 140 mg/g dw) were higher than the optimal for the ME system (5 g/L: ~125 mg/g dw). In contrast, the lower loss of mg/g made by the increase of S/L was shown on the quercetin *O*-pentoside by the ME system ($m = -0.0128$). Nevertheless, a similar behaviour was found for the MAE process ($m = -0.0163$) and the extraction yield (mg/g dw) varies in the same magnitude for both extraction techniques (~14 to 10 mg/g dw).

4. Conclusions

The combined effects of three independent variables were studied to maximize the intrinsic responses on the *J. regia* chemical composition. A 5-level full factorial Box-Behnken design of 16 combinations and 6 replicates at the centre of the experimental domain was successfully applied for the optimization of the ME and the MAE techniques by RSM. Polynomial responses were successfully designed and experimentally verified on 3-*O*-caffeoylquinic acid (P1), quercetin 3-*O*-glucoside (P2), quercetin *O*-pentoside (P3), total HPLC (sum of the most abundant compounds), total phenolics (PHE) and total flavonols (FLAV) extraction and yield maximizations. Under the global HPLC optimum conditions for the ME and the MAE systems (t = 112.5 min, T = 61.3 °C, S = 50.4% of ethanol and t = 3.0 min, T = 107.5 °C, S = 67.9% of ethanol, respectively), the values for P1, P2, P3, P1 + P2 + P3, PHE and FLAV were 10.7 and 8.6 mg/g dw, 13.2 and 15.2 mg/g dw, 12.7 and 15.1 mg/g dw, 28.3 and 32.9 mg/g dw, 29.9 and 46.4%, 129.1 and 209.1 mg/g dw, 54.7 and 66.5 mg/g dw. For the S/L at the optimum conditions, the responses for all criteria assessed followed a decreasing linear relation until 140 g/L. Statistical validation was made by the high values of the adjusted coefficient of determination. This study contributed to the valorization of walnut leaves as a source of valuable compounds. Moreover, the use of the mathematical models allowed an optimization of the target responses aimed at valorising the obtained extracts with potential application as ingredients in functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.06.012.

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