Separation and Purification Technology 192 (2018) 501–512

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Cold extraction of phenolic compounds from watercress by high hydrostatic pressure: Process modelling and optimization

José Pinela[a,b], M.A. Prieto[a,c], Lillian Barros[a], Ana Maria Carvalho[a], M. Beatriz P.P. Oliveira[b], Jorge A. Saraiva[d], Isabel C.F.R. Ferreira[a,*]

[a] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[b] REQUIMTE/LAQV, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, n° 228, 4050-313 Porto, Portugal
[c] Nutrition and Bromatology Group, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, E32004 Ourense, Spain
[d] QOPNA, Departamento de Química, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

ARTICLE INFO

Keywords:
Nasturtium officinale
Phenolic acids
Flavonoids
High hydrostatic pressure
Extraction optimization

ABSTRACT

High hydrostatic pressure (HHP) was applied to the extraction of phenolic compounds from watercress (*Nasturtium officinale*). The process was optimized by response surface methodology using a five-level central composite design combining the independent variables of processing time (*t*, 1.5–33.5 min), pressure (*P*, 0.1–600 MPa) and solvent (*S*, 0–100% of ethanol, v/v). The individual and grouped phenolic compounds, analyzed by HPLC-DAD-ESI/MS, and the extraction yield were used as response variables. The theoretical models were fitted to the experimental data, statistically validated, and used in the prediction and optimization steps. The optimal HHP conditions for the extraction of phenolic compounds were: $t = 3.1$ min, $P = 600$ MPa and $S = 100\%$, and originated 64.68 ± 2.97 mg/g of extract. This study highlighted the HHP as a promising technology to cold extract phenolic compounds (phenolic acids and flavonoids) from watercress in a selective way using a green solvent and reduced extraction times.

1. Introduction

The recovery of bioactive compounds from natural sources and their further incorporation into foods [1], dietary supplements [2] and cosmeceuticals [3], either in isolated form or in enriched extracts, is a current hot topic that involves many research fields. Phenolic compounds are among the most desired plant secondary metabolites because of their recognized bioactivities and capacity to protect against free radical-mediated diseases [4]. Several studies have been carried out in recent years to improve the extraction of these compounds from plant materials [5–7], but more efficient and sustainable methods need to be developed to achieve higher yields and superior quality products at lower processing costs.

Watercress (*Nasturtium officinale* R. Br.) is a semi-aquatic fast-growing plant of the Brassicaceae family with recognized health-promoting effects. Its consumption in a daily diet has been linked with a reduced risk of chronic diseases including different types of cancer [8–11]. This species is an interesting source of pharmacologically active phytochemicals [12–14] whose involvement in antigenotoxic and anticancer processes has been demonstrated in both *in vivo* and *in vitro* assays [11,15–17]. A previous study reported *p*-coumaric acid, quercetin-3-*O*-sophoroside and isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside as the most abundant phenolic compound in wild watercress [14]. Higher concentrations of flavonoids than phenolic acids were reported in these extracts due to the high contents of isorhamnetin and quercetin glycosides and, in lesser extent, of kaempferol [14]. In turn, a dimer of caffeoylmalic acid, disinapoylgentibiose and ferulic acid were identified as the predominant polyphenols in watercress juice, which demonstrated capacity to inhibit digestive enzymes relevant to type 2 diabetes and obesity [12]. Despite the great potential of these compounds in various industrial sectors, the development of more efficient processes for their recovery from watercress and other natural sources remains challenging.

High hydrostatic pressure (HHP) is an emerging technology increasingly used in the food industry as a cold pasteurization method [18–21]. It consists on subjecting packaged or in bulk foods to pressures up to 1000 MPa inside a vessel filled with water, fluid that acts as pressure-transmitting medium [18,21]. During processing, the pressure is transmitted in an isostatic and quasi-instantaneous manner throughout the sample, which makes the processing time independent

Abbreviations: *Igd*, isorhamnetin glycoside derivatives; *Kgd*, kaempferol glycoside derivatives; *P*, pressure; *Qgd*, quercetin glycoside derivatives; *S*, solvent; *T*, processing time

* Corresponding author.
E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

http://dx.doi.org/10.1016/j.seppur.2017.10.007
Received 7 August 2017; Received in revised form 3 October 2017; Accepted 4 October 2017
Available online 05 October 2017
1383-5866/

Table 1
Experimental design (independent variables and their coded and natural values) and values for phenolic compounds (mg/g of extract) and extraction yield (%) achieved under the 20 runs involved in the HHP extraction optimization by RSM.

Five-level CCCD experimental design																					
	Runs		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Coded values	X_1: Time (t)		−1	1	−1	1	−1	1	−1	1	−1.68	1.68	0	0	0	0	0	0	0	0	0	0
	X_2: Pressure (P)		−1	−1	1	1	−1	−1	1	1	0	0	−1.68	1.68	0	0	0	0	0	0	0	0
	X_3: Solvent (S)		−1	−1	−1	−1	1	1	1	1	0	0	0	0	−1.68	1.68	0	0	0	0	0	0
Natural values	X_1: t (min)		8	27	8	27	8	27	8	27	1.5	33.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5
	X_2: P (MPa)		122	122	478	478	122	122	478	478	300	300	0.1	600	300	300	300	300	300	300	300	300
	X_3: S (% of ethanol, v/v)		20	20	20	20	80	80	80	80	50	50	50	50	0	100	50	50	50	50	50	50
Response variables for RSM application																						
Quercetin-3-*O*-sophoroside	P1		1.30	1.24	0.99	1.06	1.13	1.10	1.21	1.25	1.17	1.16	1.17	1.12	1.01	0.99	1.13	1.13	1.14	1.13	1.13	1.16
Quercetin-3-*O*-manolylglucoside-7-*O*-glucoside	P3		1.89	1.80	1.18	1.32	1.27	1.14	2.13	1.93	1.59	1.53	1.54	1.51	1.20	1.04	1.44	1.44	1.45	1.45	1.45	1.47
Quercetin-3-*O*-rutinoside-7-*O*-glucoside	P7		0.00	0.01	0.01	0.01	0.88	0.91	0.93	0.92	0.91	0.90	0.90	0.89	0.88	0.92	0.89	0.90	0.90	0.90	0.91	0.91
Quercetin-3-*O*-rutinoside (rutin)	P10		1.05	1.03	0.99	1.06	1.09	1.11	1.05	1.07	1.11	1.10	1.13	1.15	1.06	0.96	1.19	1.20	1.21	1.20	1.20	1.20
Quercetin-*O*-sophoroside-*O*-rutinoside	P11		1.00	0.97	0.95	1.03	1.01	1.01	1.11	1.13	1.07	1.05	1.07	1.13	0.91	0.93	1.15	1.15	1.16	1.15	1.15	1.15
Quercetin-*O*-coumaroylsophoroside	P12		1.39	1.33	1.11	1.22	1.03	0.97	1.32	1.30	1.17	1.13	1.19	1.38	1.71	1.00	1.48	1.47	1.46	1.48	1.30	1.51
Quercetin-*O*-sophoroside-*O*-malonylhexoside	P13		2.14	1.96	1.57	1.99	2.24	1.94	6.09	5.13	2.98	2.74	2.73	3.33	1.39	0.93	3.29	3.15	3.22	3.30	2.82	3.29
Quercetin-*O*-dihexosyl-*O*-malonylhexoside	P14		0.95	0.89	0.87	0.89	0.92	0.91	1.05	1.01	0.94	0.92	0.93	0.93	0.86	0.00	0.94	0.95	0.95	0.94	0.93	0.99
Quercetin-*O*-sinapoylhexoside-*O*-rutinoside	P15		1.17	1.13	1.04	1.18	1.19	1.16	1.32	1.35	1.25	1.23	1.24	1.29	0.99	0.99	1.32	1.30	1.32	1.31	1.26	1.30
Total quercetin glycoside derivatives (Qgd)	–		10.88	10.36	8.70	9.76	10.77	10.24	16.20	15.09	12.20	11.76	11.90	12.72	10.01	13.76	12.83	12.68	12.79	12.85	12.15	12.96
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside	P16		1.58	1.53	1.38	1.83	1.73	1.61	2.79	2.72	2.08	1.98	2.06	2.43	1.12	1.05	2.54	2.47	2.51	2.52	2.47	2.54
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-malonylhexoside	P17		2.97	2.89	1.96	3.01	3.60	3.00	9.22	7.88	4.31	3.99	4.01	4.70	1.77	0.98	4.80	4.59	4.78	4.73	4.73	4.73
Isorhamnetin-*O*-sophoroside-*O*-malonylhexoside	P18		2.31	2.25	1.67	2.49	2.94	2.43	7.35	6.25	3.44	3.13	3.09	3.52	1.47	1.03	3.53	3.47	3.53	3.51	3.51	3.53
Total isorhamnetin glycoside derivatives (Igd)	–		6.86	6.67	5.01	7.33	8.27	7.03	19.36	16.85	9.83	9.11	9.15	10.65	4.36	8.06	10.88	10.54	10.82	10.76	10.71	10.81
Kaempferol-*O*-feruloylhexoside-*O*-rutinoside	P19		1.04	1.00	0.95	1.02	1.03	1.01	1.21	1.20	1.08	1.06	1.07	1.13	0.92	0.96	1.14	1.14	1.14	1.14	1.14	1.14
Kaempferol-*O*-feruloylhexoside-*O*-hexoside	P20		0.99	0.97	0.93	1.00	0.99	0.98	1.14	1.13	1.05	1.03	1.04	1.10	0.91	0.93	1.11	1.12	1.12	1.11	1.11	1.11
Kaempferol-*O*-hydroxyferuloylglucuronide-*O*-malonylhexoside	P21		1.27	1.24	1.06	1.26	1.42	1.31	2.24	2.12	1.48	1.43	1.43	1.52	1.03	0.93	1.53	1.51	1.54	1.52	1.51	1.54
Kaempferol-*O*-feruloylhexoside-*O*-malonylhexoside	P22		1.20	1.21	1.03	1.18	1.27	1.18	1.92	1.81	1.42	1.39	1.33	1.48	0.90	1.10	1.43	1.42	1.43	1.42	1.42	1.43
Total kaempferol glycoside derivatives (Kgd)	–		4.51	4.42	3.97	4.46	4.72	4.49	6.51	6.27	5.04	4.91	4.88	5.23	3.76	5.92	5.22	5.19	5.23	5.19	5.18	5.21
Total flavonoids	–		22.3	21.4	17.7	21.6	23.8	21.8	42.1	38.2	27.1	25.8	25.9	28.6	18.1	24.7	28.9	28.4	28.8	28.8	28.0	29.0
p-Coumaric acid hexoside	P2		0.00	0.00	0.00	0.00	0.06	0.07	0.03	0.03	0.04	0.04	0.04	0.04	0.02	0.05	0.04	0.05	0.04	0.04	0.04	0.04
Ferulic acid hexoside	P4		0.00	0.01	0.01	0.08	0.11	0.11	0.09	0.10	0.09	0.09	0.09	0.09	0.02	0.08	0.18	0.18	0.18	0.18	0.18	0.18
Caffeic acid	P5		2.63	2.62	1.39	2.15	2.21	1.91	3.32	3.13	2.68	2.58	2.64	2.44	2.00	2.05	2.56	2.40	2.51	2.51	2.50	2.48
p-Coumaric acid	P6		3.91	3.72	2.05	3.40	4.25	4.07	4.82	4.91	4.54	4.38	4.51	4.07	3.06	2.88	4.30	4.11	4.24	4.12	4.16	4.12
Ferulic acid	P8		1.07	1.12	0.59	0.96	1.33	1.28	1.80	1.72	1.39	1.28	1.31	1.19	0.95	0.17	1.23	1.18	1.21	1.18	1.17	1.22
Sinapoylmalic acid	P9		0.33	0.32	0.16	0.27	0.49	0.43	0.89	0.78	0.51	0.46	0.51	0.45	0.24	0.18	0.43	0.43	0.43	0.44	0.45	0.46
Total phenolic acids	–		7.9	7.8	4.2	6.9	8.4	7.9	11.0	10.7	9.2	8.8	9.1	8.3	6.3	10.4	8.7	8.3	8.6	8.5	8.5	8.5
Total phenolic compounds	–		30.2	29.2	21.9	28.4	32.2	29.6	53.0	48.9	36.3	34.6	35.0	36.9	24.4	33.2	37.7	36.7	37.5	37.3	36.5	37.5
Extraction yield (crude extract)	–		19.4	21.5	27.5	26.8	14.9	16.9	16.1	19.5	23.8	26.2	18.9	21.0	26.0	12.0	22.6	24.2	21.8	24.5	22.1	23.9

of the sample shape or size. In addition, the temperature increase with increasing pressure is minimal (~3 °C/100 MPa) [22,23], thus being a good alternative to heat-based treatments.

HHP has been explored for some time by the food industry. Its application for extraction of high added-value compounds from plant materials is relatively recent and very promising, but more research focusing on different compounds and plant materials is still needed. The applied pressure promotes the rupture of the plant tissues, cell walls and organelles, a phenomenon that enhances the mass transfer of the solvent into the sample and of compounds to the solvent [24]. In addition, the higher the hydrostatic pressure is, the more solvent can enter cells and the more compounds can permeate out to the solvent [25,26]. Despite the considerable cost of the HHP equipment, processing can become cheaper compared to conventional methods that demand temperature and long processing times [27]. Therefore, the equipment costs could be repaid back in long-term usage.

Previous studies reported that HHP is a good alternative to conventional extraction methods since it avoids the degradation of thermosensitive molecules, reduces the extraction time and solvent consumption, and improves the extraction efficiency in terms of yield, quality and selectivity [24–26,28–30]. Moreover, a low-energy input is required by this eco-friendly technology to compress a sample to 500 MPa as compared to heating to 100 °C [31]. As examples, HHP was successfully applied to extract antioxidant compounds from pomegranate [32] and citrus [33] peels and fig by-products [30], flavonoids from propolis [29], anthocyanins from grape skins [34], catechins [25] and caffeine [35] from green tea leaves, ginsenosides from ginseng (*Panax ginseng* C.A. Meyer) [36], ferulic acid from Radix *Angelica sinensis* [37], and carotenoids from tomato wastes [22]. However, the performance of this extraction method can be affected by a number of independent variables such as processing time, pressure and solvent [30,32], whose effect on one or more dependent (response) variables can be evaluated using the response surface methodology (RSM). This is a time- and reagent-saving statistical tool increasingly used in process optimization since one-factor-at-a-time experiments cannot predict optimal conditions and neglect interactions between variables.

The present study was carried out to optimize the HHP extraction of phenolic compounds from watercress using RSM. The response variables used in the development of mathematical models describing the extraction process (namely individual and grouped phenolic compounds) were obtained by high-performance liquid chromatography coupled to mass spectrometry (HPLC-DAD-ESI/MS).

2. Material and methods

2.1. Standards and reagents

HPLC-grade acetonitrile was purchased from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (ferulic, sinapic, *p*-coumaric and caffeic acids, and kaempferol-3-*O*-rutinoside, quercetin-3-*O*-rutinoside, and quercetin-3-*O*-glucoside) were purchased from Extrasynthese (Genay, France). All other chemicals were of analytical grade and were purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

2.2. Plant material

Fresh samples of watercress (*Nasturtium officinale* R. Br.) were commercially obtained from a local supermarket in Bragança, Portugal. The taxonomic identification of the plant material was confirmed by the botanist Dr. Ana Maria Carvalho from the Polytechnic Institute of Bragança, Portugal. The samples were lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine powder (~20 mesh), and kept at −20 °C until processing.

2.3. High hydrostatic pressure extraction

The extractions were carried out on a pilot-scale high-pressure equipment (Model 55, Hyperbaric, Burgos, Spain) with a pressure vessel of 55 L, connected to a refrigeration unit (RMA KH 40 LT, Ferroli, San Bonifacio, Italy) to control the temperature of the input water used as pressure-transmitting fluid. Heat-sealed plastic bags containing 0.6 g of dry powder sample and 20 mL of solvent were placed in the pressure vessel and then subjected to different conditions of processing time (1.5–33.5 min), pressure (0.1–600 MPa) and solvent (0–100% of ethanol, v/v) as defined in the circumscribed central composite design (CCCD) presented in Table 1. Ethanol:water mixtures were used since ethanol has low toxicity and GRAS (generally recognized as safe) status. The solid/liquid ratio was maintained at 30 g/L. All extractions were performed at 20 °C (cold extraction). However, since the pressure increases the temperature by ~3 °C/100 MPa [22,23], processing at 600 MPa resulted in an adiabatic temperature increase from 20 °C to ~38 °C, which should still be not enough to promote the thermal degradation of bioactive compounds. After HHP processing, the mixture was filtered through filter paper (Whatman No. 4) and the filtrate was collected and kept at −80 °C until analysis.

2.4. Calculation of the extraction yield

The extraction yields (%) were calculated based on the dry weight (crude extract) obtained after evaporation of the solvent. First, the filtrates were concentrated at 35 °C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and the aqueous phase was then lyophilised to obtain a dried extract.

2.5. Chromatographic analysis of phenolic compounds

The dried extracts (~10 mg) were dissolved in a methanol:water mixture (20:80 v/v) and filtered through 0.22 μm disposable LC filter disks. The chromatographic analysis was performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector (DAD) coupled to an electrospray ionization mass detector (ESI-MS) (ThermoFinnigan, San Jose, CA, USA) as described by Bessada et al. [38]. The phenolic compounds were identified using 280 nm and 370 nm as preferred wavelengths and by comparing their retention time and UV–vis and mass spectra with those obtained from authentic standards, when available. For quantitative analysis, a baseline to valley integration with baseline projection mode was used to calculate the peak areas and the external standards mentioned above were used for quantification. The results were expressed in mg per g of extract.

2.6. Experimental design, modelling and optimization

2.6.1. Experimental design

A five-level CCCD (Box-Behnken design) coupled with RSM was implemented to optimize the HHP conditions for the extraction of phenolic compounds from watercress. The coded and natural values of the independent variables X_1 (processing time (*t*), min), X_2 (pressure (*P*), MPa) and X_3 (solvent (*S*), % of ethanol, v/v) are presented in Table 1. This CCCD includes 6 replicated center points and a group of axial points chosen to allow rotatability, which ensures that the variance of the model prediction is constant at all points equidistant from the design center. The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

2.6.2. Mathematical modelling

The response surface models were fitted by means of least-squares calculation using the following Box-Behnken design equation:

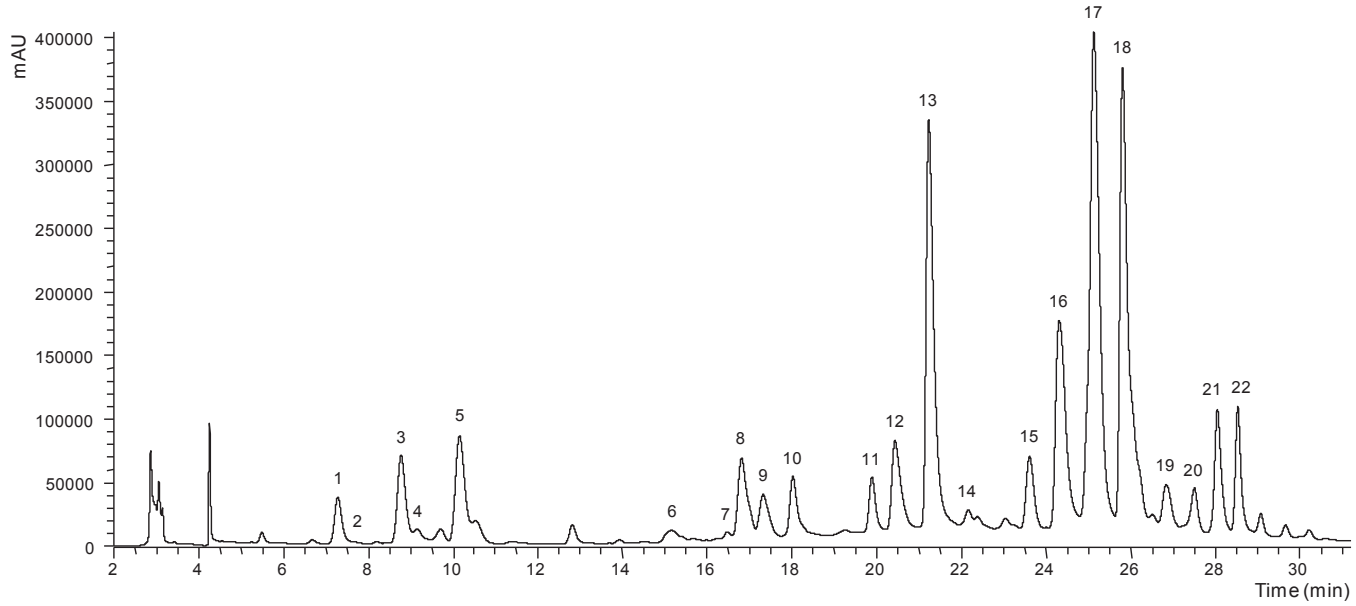

Fig. 1. HPLC profile of phenolic compounds of the watercress extract obtained under the experimental run No. 20, recorded at 370 nm. See Table 1 for peak identification.

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

In this equation, Y represents the dependent variable (response variable) to be modelled, X_i and X_j are the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} is the coefficient of quadratic effect, and n is the number of variables. The extraction yield and the individual and grouped phenolic compounds (22 compounds and 6 groups) were used as dependent variables.

2.6.3. Procedure to optimize the variables to a maximum response

A simplex method was used to optimize the predictive model by solving nonlinear problems in order to maximize the extraction yield and the recovery of phenolic compounds [5]. Certain limitations were imposed (*i.e.*, times lower than 0) to avoid variables with unnatural and unrealistic physical conditions.

2.7. Cluster analyses

A cluster analysis was performed to group the phenolic compounds according to the extraction conditions that maximize their response values using the "XLSTAT 2016", a Microsoft Excel add-in. A comparative agglomerative hierarchical clustering analysis (HCA) with Pearson correlation coefficient was used for clustering (similarity analysis). The algorithm used was a complete linkage with automatic truncation based on entropy.

2.8. Fitting procedures and statistical analysis

Fitting procedures, coefficient estimates and statistical calculations were performed as previously described by Pinela et al. [5]. In brief, a) the coefficient measurement was performed using the nonlinear least-square (quasi-Newton) method provided by the macro "Solver" in Microsoft Excel, which allows minimizing the sum of the quadratic differences between the observed and model-predicted values; b) the coefficient significance was evaluated using the 'SolverAid' to determine the parametric confidence intervals. The not statistically significant terms (p-value > 0.05) were dropped to simplify the model; and c) the model reliability was verified using the following criteria: i) the Fisher F-test ($\alpha = 0.05$) was used to determine whether the constructed models were adequate to describe the observed data; ii) the 'SolverStat' macro was used for the assessment of parameter and model prediction uncertainties; iii) the R^2 was interpreted as the proportion of variability of the dependent variable explained by the model.

3. Results and discussion

3.1. Response criteria for the RSM analysis

The experimental values achieved for the 20 experimental runs of the CCCD design are presented in Table 1. The HPLC phenolic profile (recorded at 370 nm) of the watercress extract obtained under the experimental run No. 20 is shown in Fig. 1 (see HHP extraction conditions in Table 1). This profile is concordant with that previously characterized by Pinela et al. [14] for wild watercress. Up to twenty-two compounds were identified (Table 1) based on their chromatographic, UV–vis and mass spectra characteristics, six of which were phenolic acid derivatives (hydroxycinnamic acids) and sixteen were flavonoid glycoside derivatives. Many of these compounds were also reported by other authors in this species [12,39,40]. Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-malonylhexoside, *p*-coumaric acid, isorhamnetin-*O*-sophoroside-*O*-malonylhexoside, quercetin-*O*-sophoroside-*O*-malonylhexoside and caffeic acid were identified as the most abundant compounds. Flavonoids predominated over phenolic acids and, in general, more quercetin and isorhamnetin glycoside derivatives were quantified than phenolic acids.

For optimization purposes, the phenolic compounds quantified by chromatographic methods (Table 1) were grouped in total phenolic acids (compounds 2, 4, 5, 6, 8 and 9), total flavonoids, comprising the subgroups of quercetin glycoside derivatives (*Qgd*: compounds 1, 3, 7, 10, 11, 12, 13, 14 and 15), isorhamnetin glycoside derivatives (*Igd*: compounds 16, 17 and 18) and kaempferol glycoside derivatives (*Kgd*: compounds 19, 20, 21 and 22), and total phenolic compounds (including all quantified phenolics). The individual and grouped compounds were used as response criteria to optimize the HHP conditions for their extraction from watercress using RSM. The values of the extraction yield were also considered, which ranged from 12 to 27.5% with the experimental runs n° 14 and 3, respectively (Table 1). Therefore, a total of 29 response variables were computed and used as optimization criteria.

Table 2
Fitting coefficients and R^2 determined for the models obtained for individual and grouped phenolic compounds and extraction yield (Table 3), and optimal HHP conditions and response values.

Response variables	Fitting coefficients obtained after applying the Box-Behnken design equation						
	Intercept	Linear effect			Quadratic effect		
	b_0	b_1 (t)	b_2 (P)	b_3 (S)	b_{11} (t^2)	b_{22} (P^2)	b_{33} (S^2)
Compound P1	1.14 ± 0.01	ns	−0.02 ± 0.01	ns	0.02 ± 0.01	0.02 ± 0.01	−0.04 ± 0.01
Compound P3	1.51 ± 0.05	−0.03 ± 0.00	ns	ns	ns	0.06 ± 0.04	−0.09 ± 0.04
Compound P7	0.90 ± 0.07	ns	0.02 ± 0.01	0.02 ± 0.01	ns	ns	ns
Compound P10	1.20 ± 0.01	ns	ns	ns	−0.04 ± 0.01	−0.03 ± 0.01	−0.07 ± 0.01
Compound P11	1.15 ± 0.01	ns	0.02 ± 0.01	0.03 ± 0.01	−0.03 ± 0.01	−0.02 ± 0.01	−0.08 ± 0.01
Compound P12	1.45 ± 0.04	ns	0.04 ± 0.03	−0.12 ± 0.03	−0.12 ± 0.03	−0.07 ± 0.03	−0.04 ± 0.03
Compound P13	3.00 ± 0.10	−0.12 ± 0.12	0.57 ± 0.12	0.95 ± 0.13	ns	ns	ns
Compound P14	0.94 ± 0.01	−0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.01	ns	−0.01 ± 0.01	ns
Compound P15	1.29 ± 0.02	ns	0.02 ± 0.01	0.04 ± 0.01	−0.01 ± 0.01	ns	−0.10 ± 0.01
Total Qgd	12.52 ± 0.26	ns	0.65 ± 0.19	1.39 ± 0.23	−0.35 ± 0.03	ns	−0.37 ± 0.24
Compound P16	2.51 ± 0.09	ns	0.21 ± 0.06	0.18 ± 0.06	−0.14 ± 0.06	−0.06 ± 0.06	−0.47 ± 0.06
Compound P17	4.46 ± 0.16	−0.16 ± 0.13	0.85 ± 0.19	1.53 ± 0.21	ns	ns	ns
Compound P18	3.44 ± 0.14	ns	0.63 ± 0.15	1.28 ± 0.20	ns	ns	ns
Total Igd	10.74 ± 0.27	−0.15 ± 0.03	1.72 ± 0.27	1.62 ± 0.27	ns	ns	−1.84 ± 0.27
Compound P19	1.13 ± 0.01	ns	0.03 ± 0.01	0.04 ± 0.01	−0.02 ± 0.01	ns	−0.07 ± 0.01
Compound P20	1.11 ± 0.01	ns	0.03 ± 0.01	0.03 ± 0.01	−0.02 ± 0.01	−0.01 ± 0.01	−0.07 ± 0.01
Compound P21	1.50 ± 0.02	ns	0.12 ± 0.03	0.28 ± 0.03	ns	ns	ns
Compound P22	1.44 ± 0.03	ns	0.10 ± 0.03	0.14 ± 0.03	ns	ns	−0.13 ± 0.03
Total Kgd	5.20 ± 0.03	ns	0.27 ± 0.03	0.58 ± 0.03	−0.08 ± 0.01	−0.05 ± 0.03	−0.16 ± 0.03
Total flavonoids	28.68 ± 0.84	ns	2.55 ± 0.51	5.23 ± 0.63	−0.91 ± 0.52	−0.61 ± 0.52	−0.85 ± 0.65
Compound P2	0.04 ± 0.01	ns	−0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	ns	−0.01 ± 0.01
Compound P4	0.18 ± 0.01	0.01 ± 0.00	ns	0.03 ± 0.00	−0.03 ± 0.01	−0.04 ± 0.01	−0.05 ± 0.00
Compound P5	2.54 ± 0.05	ns	ns	0.13 ± 0.05	ns	ns	−0.15 ± 0.05
Compound P6	4.19 ± 0.07	ns	−0.11 ± 0.07	0.55 ± 0.07	ns	ns	−0.19 ± 0.07
Compound P8	1.20 ± 0.03	ns	ns	0.28 ± 0.02	0.03 ± 0.02	ns	0.04 ± 0.02
Compound P9	0.45 ± 0.02	ns	0.04 ± 0.02	0.17 ± 0.02	ns	ns	0.03 ± 0.01
Total phenolic acids	8.56 ± 0.16	ns	ns	1.27 ± 0.17	ns	ns	−0.26 ± 0.18
Total phenolic compounds	37.23 ± 1.02	ns	2.49 ± 0.62	6.49 ± 0.76	−0.85 ± 0.13	−0.66 ± 0.63	−1.11 ± 0.80
Extraction yield	23.90 ± 0.62	0.79 ± 0.49	1.52 ± 0.49	−4.00 ± 0.49	ns	−0.97 ± 0.47	−3.45 ± 0.47

Response variables	Fitting coefficients obtained after applying the Box-Behnken design equation				Optimal processing conditions and response values			
	Interactive effect			R^2	t (min)	P (MPa)	S (%)	Optimum
	b_{12} (tP)	b_{13} (tS)	b_{23} (PS)					
Compound P1	0.03 ± 0.01	ns	0.09 ± 0.01	0.8649	1.5 ± 0.1	0.0 ± 6.7	0.0 ± 9.7	1.50 ± 0.52
Compound P3	ns	−0.05 ± 0.05	0.36 ± 0.05	0.8073	1.5 ± 0.1	600.0 ± 3.0	100.0 ± 5.1	2.61 ± 0.87
Compound P7	−0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.9093	1.5 ± 0.1	600.0 ± 1.5	100.0 ± 0.2	0.98 ± 0.32
Compound P10	ns	ns	ns	0.8310	17.5 ± 0.3	300.0 ± 4.5	50.0 ± 0.7	1.20 ± 0.17
Compound P11	0.02 ± 0.01	ns	0.03 ± 0.01	0.9490	21.1 ± 0.2	521.4 ± 4.8	61.4 ± 0.6	1.17 ± 0.39
Compound P12	ns	ns	0.12 ± 0.04	0.7933	17.5 ± 0.8	75.3 ± 3.3	0.0 ± 0.0	1.64 ± 0.55
Compound P13	ns	ns	0.91 ± 0.16	0.8956	1.5 ± 0.2	600.0 ± 6.3	100.0 ± 10.4	8.31 ± 2.35
Compound P14	ns	ns	0.04 ± 0.01	0.8187	1.5 ± 0.1	600.0 ± 3.8	100.0 ± 0.6	1.13 ± 0.58
Compound P15	0.03 ± 0.02	ns	0.05 ± 0.02	0.8682	33.5 ± 0.6	600.0 ± 9.1	67.3 ± 1.1	1.41 ± 0.51
Total Qgd	ns	ns	1.63 ± 0.24	0.8993	17.5 ± 0.9	600.0 ± 13.4	100.0 ± 5.6	19.53 ± 2.18
Compound P16	ns	ns	0.26 ± 0.08	0.8736	17.5 ± 1.7	600.0 ± 5.7	69.3 ± 6.5	2.88 ± 0.73
Compound P17	ns	ns	1.26 ± 0.24	0.8952	1.5 ± 0.3	600.0 ± 9.8	100.0 ± 6.4	12.30 ± 2.86
Compound P18	ns	−0.30 ± 0.20	1.08 ± 0.20	0.9031	1.5 ± 0.2	600.0 ± 8.6	100.0 ± 4.3	10.54 ± 2.65
Total Igd	ns	−0.82 ± 0.14	2.24 ± 0.27	0.8926	1.5 ± 0.1	600.0 ± 11.8	97.1 ± 24.3	16.89 ± 2.26

Table 2 *(continued)*

Response variables	Fitting coefficients obtained after applying the Box-Behnken design equation				Optimal processing conditions and response values			
	Interactive effect			R^2	*t* (min)	*P* (MPa)	*S* (%)	Optimum
	b_{12} (*tP*)	b_{13} (*tS*)	b_{23} (*PS*)					
Compound P19	0.01 ± 0.01	ns	0.05 ± 0.01	0.8928	24.5 ± 0.3	600.0 ± 6.9	79.1 ± 0.9	1.26 ± 0.48
Compound P20	0.01 ± 0.01	ns	0.04 ± 0.01	0.9213	22.2 ± 0.3	600.0 ± 6.3	71.8 ± 0.8	1.16 ± 0.39
Compound P21	ns	−0.05 ± 0.03	0.23 ± 0.03	0.9300	1.5 ± 0.1	600.0 ± 9.2	100.0 ± 2.2	2.95 ± 1.40
Compound P22	ns	−0.04 ± 0.04	0.19 ± 0.04	0.8466	1.5 ± 0.1	600.0 ± 9.3	100.0 ± 3.2	2.11 ± 0.79
Total Kgd	0.07 ± 0.01	−0.11 ± 0.04	0.51 ± 0.03	0.9603	13.7 ± 0.4	600.0 ± 16.4	100.0 ± 2.7	7.49 ± 0.88
Total flavonoids	ns	−1.11 ± 0.66	4.90 ± 0.66	0.9357	7.8 ± 0.5	600.0 ± 5.0	100.0 ± 8.6	52.45 ± 2.63
Compound P2	ns	ns	−0.02 ± 0.01	0.8848	17.5 ± 0.1	298.0 ± 1.1	97.3 ± 0.4	0.06 ± 0.08
Compound P4	0.02 ± 0.01	−0.01 ± 0.01	−0.01 ± 0.01	0.9659	18.0 ± 0.1	289.9 ± 1.7	59.5 ± 0.3	0.19 ± 0.14
Compound P5	0.08 ± 0.06	−0.12 ± 0.06	0.47 ± 0.06	0.8725	1.5 ± 0.1	600.0 ± 3.6	100.0 ± 5.1	3.79 ± 0.60
Compound P6	0.23 ± 0.09	−0.16 ± 0.09	0.45 ± 0.09	0.8825	1.5 ± 0.1	0.0 ± 9.7	54.7 ± 4.1	5.02 ± 0.96
Compound P8	0.03 ± 0.01	−0.07 ± 0.03	0.19 ± 0.03	0.9176	1.5 ± 0.1	600.0 ± 8.3	100.0 ± 3.1	2.51 ± 0.68
Compound P9	ns	−0.02 ± 0.01	0.10 ± 0.02	0.9026	1.5 ± 0.1	600.0 ± 9.6	100.0 ± 1.8	1.21 ± 0.60
Total phenolic acids	0.39 ± 0.18	−0.42 ± 0.18	1.25 ± 0.18	0.9107	1.5 ± 0.3	600.0 ± 9.7	100.0 ± 6.2	13.58 ± 1.99
Total phenolic compounds	ns	−1.53 ± 0.81	6.15 ± 0.81	0.9350	3.0 ± 3.2	600.0 ± 6.3	100.0 ± 2.2	64.68 ± 2.97
Extraction yield	ns	ns	−1.21 ± 0.63	0.9142	33.5 ± 0.8	530.6 ± 3.3	26.1 ± 6.2	27.82 ± 2.26

ns: non-significant coefficient; R^2: Correlation coefficient.

3.2. Theoretical response surface models

As in many research fields, when trying to develop theoretical models to predict and comprehend the effects of independent variables on certain response variables, it is necessary to evaluate its precision by fitting these models to the experimental values. In this study, the response values (Table 1) were fitted to a second-order polynomial model using a nonlinear algorithm (least-squares estimations) to develop mathematical models for each response criteria (Table 2). Table 3 shows the estimated coefficient values obtained from the polynomial model of Eq. (1) and the coefficient of correlation (R^2) for each parametric response of the extraction process. These parametric values translate the response patterns and show the complexity of the possible interactions between variables. However, not all the parameters of Eq. (1) were used for building the model since some coefficients were non-significant (*ns*). The significant ones were assessed at a 95% confidence level ($\alpha = 0.05$). The statistic lack of fit, used to test the adequacy of the obtained models, demonstrated that no considerable improvement was achieved by the inclusion of the statistically *ns* parametric values. The resulting models for each of the 29 assessed responses are presented in Table 2. In all cases, R^2 coefficients higher than 0.79 were obtained (Table 3), which indicates that the percentage of variability of each response can be explained by the model. These workable models were applied in the subsequent prediction and optimization steps, showing a good agreement between the experimental and predicted values, which indicates that the variation is explained by the independent variables.

The obtained model coefficients (Table 3) are empirical and cannot be associated with physical or chemical significance. However, they are useful for predicting the results of untested extraction conditions [41]. The sign of the effect marks the performance of the response. In this way, when a factor has a positive effect, the response is higher at the high level, and when a factor has a negative effect, the response is lower at the high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable. Based on the mathematical expressions (Table 2), no associations were found between the response variables of phenolic acids, flavonoids, quercetin glycoside derivatives (*Qgd*), isorhamnetin glycoside derivatives (*Igd*) and kaempferol glycoside derivatives (*Kgd*). However, certain features regarding the general effects of the variables are displayed. The relevance of the significant parametric values can be order as a function of the variables involved in a decreasing form as $S > P \gg t$. Alexandre et al. [32] also found *S* as the most relevant variable on the HHP extraction of bioactive compounds from pomegranate (*Punica granatum* L.) peels. Regarding the linear, quadratic, and interactive parametric effects of the developed equations, it was found that they play an important and significant role in all evaluated responses. For the linear effect, the variables *P* and *S* had strong values; meanwhile, the effect of *t* was negligible in almost all cases. All independent variables had moderate quadratic or nonlinear effects. Regarding the interactive effects, the interactions of the variable *t* with the other variables (*tP* and *tS*) were of minor relevance; meanwhile, the *PS* interaction had a strong significance in describing the behavior of almost all responses (with the exception of compound 10). The interactive parametric values of *PS* were accentuated in the responses of flavonoids, *Qgd*, *Igd*, phenolic acids, and total phenolic compounds. To make the combined effects more explicit and to visually describe the extraction trends, the results were presented in the response surface plots discussed below.

3.3. Effect of the independent variables on the target responses and optimal extraction conditions

Although parametric results can depict the patterns of the responses, 3D and 2D graphical representations may aid on their comprehension. Fig. 2 shows the response surface plots of extraction yield and grouped phenolic compounds (total phenolic acids, total flavonoids

Table 3
Mathematical models of the extraction process derived from the second-order polynomial model with interactions of Eq. (1).

Quercetin-3-*O*-sophoroside	$Y_{P1} = 1.14 - 0.02P + 0.02t^2 + 0.02P^2 - 0.04S^2 + 0.09tP + 0.09PS$	Eq. (2)
Quercetin-3-*O*-manolylglucoside-7-*O*-glucoside	$Y_{P3} = 1.51 - 0.03t + 0.06P^2 - 0.09S^2 - 0.05tS + 0.36PS$	Eq. (3)
Quercetin-3-*O*-rutinoside-7-*O*-glucoside	$Y_{P7} = 0.90 + 0.02P + 0.02S^2 - 0.02tP + 0.02tS + 0.02PS$	Eq. (4)
Quercetin-3-*O*-rutinoside (rutin)	$Y_{P10} = 1.20 - 0.04t^2 - 0.03P^2 - 0.07S^2$	Eq. (5)
Quercetin-*O*-sophoroside-*O*-rutinoside	$Y_{P11} = 1.15 + 0.02P + 0.03S - 0.03t^2 - 0.23P^2 - 0.02S^2 - 0.08tP + 0.02tS$	Eq. (6)
Quercetin-*O*-coumaroylsophoroside	$Y_{P12} = 1.45 + 0.04P - 0.12S - 0.12t^2 - 0.07P^2 - 0.04S^2 + 0.12PS$	Eq. (7)
Quercetin-*O*-sophoroside-*O*-malonylhexoside	$Y_{P13} = 3.00 - 0.12t + 0.57P + 0.95S$	Eq. (8)
Quercetin-*O*-dihexosyl-*O*-malonylhexoside	$Y_{P14} = 0.94 - 0.01t + 0.01P + 0.04S - 0.01P^2 + 0.04PS$	Eq. (9)
Quercetin-*O*-sinapoylhexoside-*O*-rutinoside	$Y_{P15} = 1.29 + 0.02P + 0.04S - 0.01t^2 - 0.10S^2 - 0.03tP + 0.05PS$	Eq. (10)
Total quercetin glycoside derivatives (Qgd)	$Y_{Qgd} = 12.52 + 0.65P + 1.39S - 0.35t^2 - 0.37S^2 + 1.63PS$	Eq. (11)
Isorhamnetin-O-hydroxyferuloylhexoside-O-hexoside	$Y_{P16} = 2.51 + 0.21P + 0.18S - 0.14t^2 - 0.06P^2 - 0.47S^2 + 0.26PS$	Eq. (12)
Isorhamnetin-O-hydroxyferuloylhexoside-O-malonylhexoside	$Y_{P17} = 4.46 - 0.16t + 0.85P + 1.53S + 1.26PS$	Eq. (13)
Isorhamnetin-O-sophoroside-O-malonylhexoside	$Y_{P18} = 3.44 + 0.63P + 1.28S - 0.30tS + 1.08PS$	Eq. (14)
Total isorhamnetin glycoside derivatives (Igd)	$Y_{Igd} = 10.74 - 0.15t + 1.72P + 1.62S - 1.84S^2 - 0.82tS + 2.24PS$	Eq. (15)
Kaempferol-O-feruloylhexoside-O-rutinoside	$Y_{P19} = 1.13 + 0.03P + 0.04S - 0.02t^2 - 0.07S^2 + 0.02tP + 0.05PS$	Eq. (16)
Kaempferol-O-feruloylhexoside-O-hexoside	$Y_{P20} = 1.11 + 0.03P + 0.03S - 0.02t^2 - 0.01P^2 - 0.07S^2 + 0.02tP + 0.04PS$	Eq. (17)
Kaempferol-O-hydroxyferuloylglucuronide-O-malonylhexoside	$Y_{P21} = 1.50 + 0.12P + 0.28S - 0.05tS + 0.23PS$	Eq. (18)
Kaempferol-O-feruloylhexoside-O-malonylhexoside	$Y_{P22} = 1.44 + 0.10P + 0.14S - 0.13S^2 - 0.04tS + 0.19PS$	Eq. (19)
Total kaempferol glycoside derivatives (Kgd)	$Y_{Kgd} = 5.20 + 0.27P + 0.58S - 0.08t^2 - 0.05P^2 - 0.16S^2 + 0.07tP - 0.11tS + 0.51PS$	Eq. (20)
Total flavonoids	$Y_{Fl} = 28.68 + 2.55P + 5.23S - 0.91t^2 - 0.61P^2 - 0.85S^2 - 1.11tS + 4.90PS$	Eq. (21)
p-Coumaric acid hexoside	$Y_{P2} = 0.04 - 0.01P + 0.02S + 0.02t^2 - 0.01S^2 - 0.02PS$	Eq. (22)
Ferulic acid hexoside	$Y_{P4} = 0.18 + 0.01t + 0.03S - 0.03t^2 - 0.04P^2 - 0.05S^2 + 0.02tP - 0.01tS - 0.01PS$	Eq. (23)
Caffeic acid	$Y_{P5} = 2.54 + 0.13S - 015S^2 + 0.08tP - 0.12tS + 0.47PS$	Eq. (24)
p-Coumaric acid	$Y_{P6} = 4.19 - 0.11P + 0.55S - 0.19S^2 + 0.23tP - 0.16tS + 0.45PS$	Eq. (25)
Ferulic acid	$Y_{P8} = 1.20 + 0.28S - 0.03t^2 - 0.04S^2 - 0.03tP - 0.07tS + 0.19PS$	Eq. (26)
Sinapoylmalic acid	$Y_{P9} = 0.45 + 0.04P + 0.17S - 0.03S^2 - 0.01tS + 0.10PS$	Eq. (27)
Total phenolic acids	$Y_{Pa} = 8.56 + 1.27S - 0.26S^2 + 0.39tP - 0.42tS + 1.25PS$	Eq. (28)
Total phenolic compounds	$Y_{Ph} = 37.23 + 2.49P + 6.49S - 0.85t^2 - 0.66P^2 - 1.11S^2 - 1.53tS + 6.15PS$	Eq. (29)
Extraction yield (crude extract)	$Y_{EY} = 23.90 + 0.79t + 1.52P - 4.01S - 0.97P^2 - 3.45S^2 + 1.21PS$	Eq. (30)

and total phenolic compounds) as well as their statistical analysis. Fig. 3 illustrates in a similar way the results for *Qgd*, *Igs* and *Kgd*. Both Figs. 2 and 3 are divided in three subsections: i) the subsection A illustrates the 3D response surface plots, whose grid surfaces were predicted with the respective second-order polynomial model described by Eq. (1) using the theoretical values presented in Table 3. For representation of these binary combinations, the excluded variable was positioned at the optimum of their experimental domain (Table 3); ii) the subsection B illustrates the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable; and iii) the subsection C shows the individual 2D responses and the optimum values (⊙). In each plot, each independent variable was positioned at the optimal value of the other two variables.

Observing the response surface plots of the extraction yield (Fig. 2), it is possible to verify that the amount of extracted material increases to an optimum value and then, in most cases, it decreases as a function of the involved independent variable. Consequently, the optimum value can be found as being a single point in almost all combinations, which allows computing the extraction conditions that lead to an absolute maximum. Fig. 2C simplifies the interpretation of the effects of the independent variables on the extraction process and highlights the optimum value of each variable. The extraction yield was maximal (27.82 ± 2.26%) when the optimal HHP conditions (t = 33.5 min, P = 530.6 MPa and S = 26.1% of ethanol, v/v) presented in Table 3 were applied for extraction. Zhang et al. [42] have shown that the crude extract obtained from *Rhodiola sachalinensis* is greater when HHP is used than when the extraction is done by the conventional methods of reflux or Soxhlet. High extraction yields were also achieved by Prasad et al. [26] when processing longan fruit (*Dimpcarpus longan* Lour.) pericarps under pressures up to 500 MPa and using lower extraction times than those required in a conventional extraction.

The response surface plots of grouped phenolic acids and flavonoids and total phenolic compounds are showed in Fig. 2 and the optimal HHP conditions that maximize their recovery from watercress are presented in Table 3. These responses were similarly affected by the screened variables; they were favoured by high values of P and S and short values of t as summarized below:

- For phenolic acids, the optimal HHP conditions were: t = 1.5 ± 0.3 min, P = 600.0 ± 5.0 MPa and S = 100.0 ± 8.6% of ethanol (v/v), and originated 13.58 ± 1.99 mg/g of extract.
- For flavonoids, the optimal HHP conditions were: t = 7.8 ± 0.5 min, P = 600.0 ± 5.0 MPa and S = 100.0 ± 8.6% of ethanol (v/v), and originated 52.45 ± 2.63 mg/g of extract.
- For total phenolic compounds, the optimal HHP conditions were: t = 3.1 ± 3.2 min, P = 600.0 ± 6.3 MPa and S = 100.0 ± 2.2 of ethanol (v/v), and originated 64.68 ± 2.97 mg/g of extract.

The optimum extraction values for the flavonoid derivatives *Qgd*, *Igs* and *Kgd* were achieved using very similar HHP conditions (Fig. 3 and Table 3), probably due to structural similarities between these compounds. Once more, the extraction was favoured by high values of P and S and short values of t, as summarized below:

- For *Qgd*, the optimal HHP conditions were: t = 17.5 ± 1.0 min, P = 600.0 ± 13.4 MPa and 100.0 ± 5.6% of ethanol (v/v), and originated 19.53 ± 2.18 mg/g of extract.
- For *Igd*, the optimal HHP conditions were: t = 1.5 ± 0.1 min, P = 600.0 ± 11.8 MPa and 97.1 ± 24.3% of ethanol, and originated 16.89 ± 2.26 mg/g of extract.
- For *Qgd*, the optimal HHP conditions were: t = 13.7 ± 0.4 min, P = 600.0 ± 16.4 MPa and S = 100.0 ± 2.7% of ethanol (v/v), and originated 7.49 ± 0.88 mg/g of extract.

According to the literature, the use of high pressures increases the extraction of bioactive compounds from plants matrices [30]. Briones-Labarca et al. [28] demonstrated that HHP is more effective than

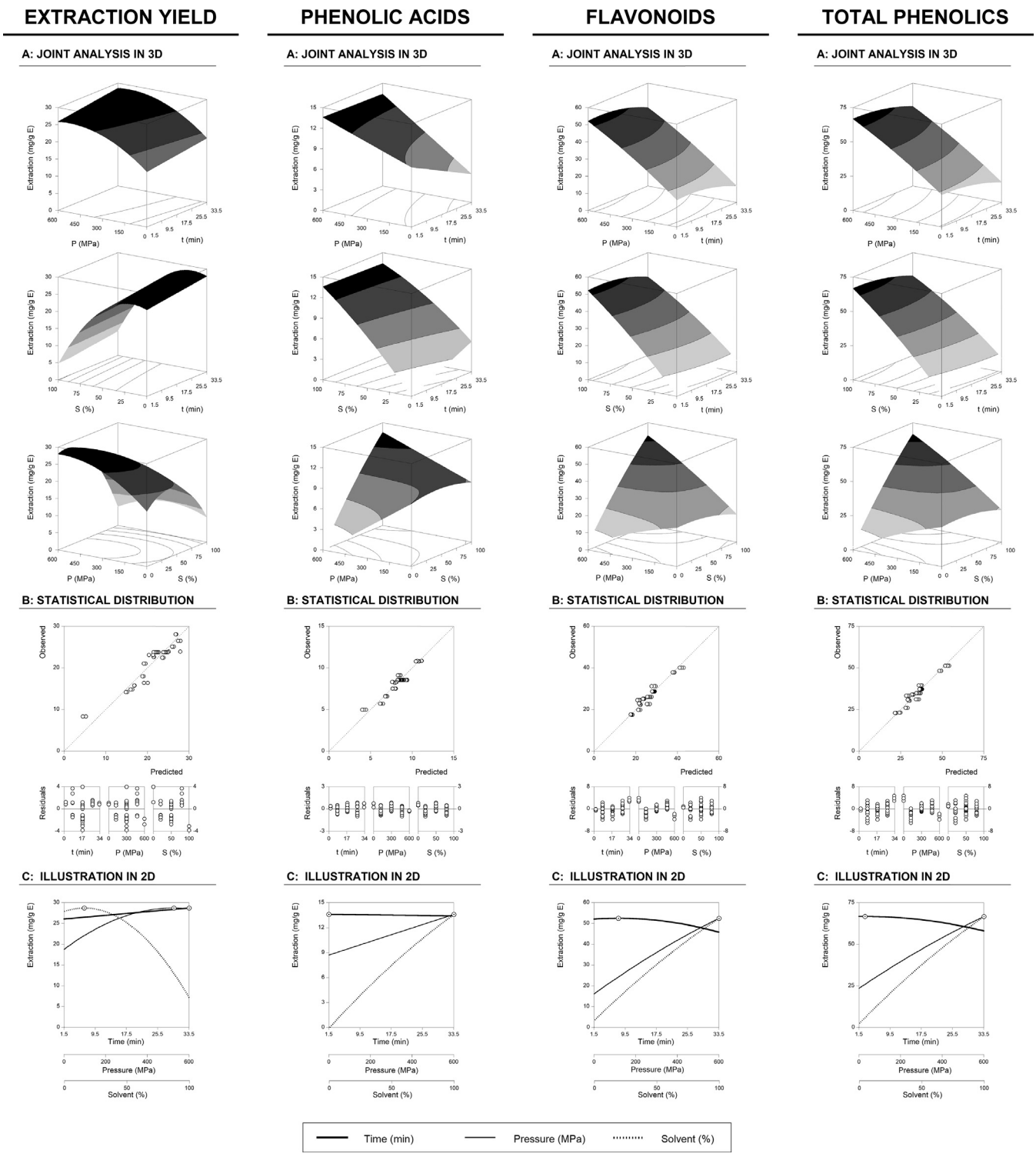

Fig. 2. Response surface plots of extraction yield and grouped phenolic compounds (total phenolic acids, total flavonoids and total phenolic compounds). Part A: 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (Table 3) predicted with Eq. (1). For representation purposes, the excluded variable was positioned at the optimum of their experimental domain (Table 3). Part B: illustration of the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable. Part C: individual 2D responses and optimum values (⊙). Each independent variable was positioned at the optimal value of the other two variables.

ultrasound-assisted extraction or conventional extraction (2 h) to recover antioxidants and total phenolic compounds from Chilean papaya (*Vasconcellea pubescens*) seeds. In addition, HHP was a time-saving extraction method. The lower energy consumption is another advantage of HHP comparatively to conventional methods [35]. In our study, it is also interesting to note that the HHP conditions that maximize the yield of crude extract and the recovery of phenolic compounds differ mostly in the required processing time and ethanol concentration. In this way, the extracts obtained under the optimal conditions established for phenolic compounds (Table 3) will contain a lower quantity of compounds other than phenolics, thus making the recovery process more selective for the target compounds.

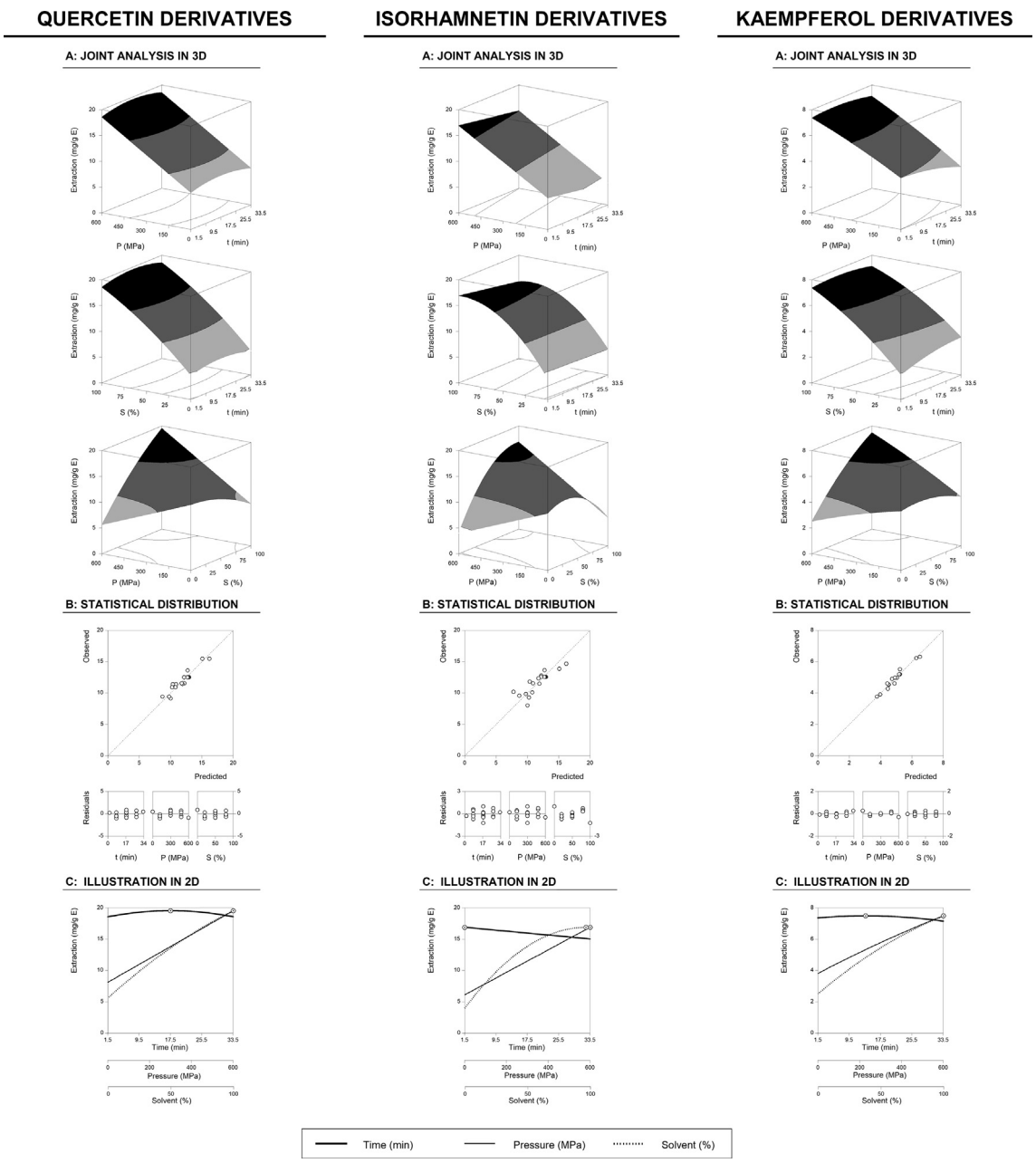

Fig. 3. Response surface plots of the flavonoid subgroups of quercetin, isorhamnetin and kaempferol glycoside derivatives. Part A: 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (Table 3) predicted with Eq. (1). For representation purposes, the excluded variable was positioned at the optimum of their experimental domain (Table 3). Part B: illustration of the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable. Part C: individual 2D responses and optimum values (⊙). Each independent variable was positioned at the optimal value of the other two variables.

The effects of the independent variables on the extraction of individual phenolic compounds from watercress are 2D represented in Fig. 4. The processing conditions that generated optimal response values (⊙) are numerically described in Table 3. The identified flavonoids were organized as a function of the maximum amount achieved (mg/g of extract) in a decreasing order as follows: P17 (12.3 ± 2.86) > P18 (10.54 ± 2.65) > P13 (8.31 ± 2.35) ≫ P21 (2.95 ± 1.40) > P16 (2.88 ± 0.73) > P3 (2.61 ± 0.87) > P22 (2.11 ± 0.79) > P12 (1.64 ± 0.55) > P1 (1.5 ± 0.52) > P15 (1.41 ± 0.51) > P19 (1.26 ± 0.48) > P10 (1.2 ± 0.17) > P11 (1.17 ± 0.39) > P20 (1.16 ± 0.39) > P14 (1.13 ± 0.58) > P7 (0.98 ± 0.32). Meanwhile, the phenolic acids were organized as follows: P6 (5.02 ± 0.96) > P5 (3.79 ± 0.60) ≫ P8 (2.51 ± 0.68) > P9 (1.21 ± 0.60) > P4 (0.19 ± 0.14) > P2 (0.06 ± 0.08). Pinela et al. [14] reported lower quantities of phenolic acids (5.6 ± 0.5 mg/g of extract), flavonoids (22 ± 1 mg/g of extract) and total phenolic compounds (28 ± 2 mg/g of extract) in an extract of wild watercress obtained by a conventional solid-liquid extraction of 2 h and using a methanol:water mixture (80:20, v/v) as a extraction solvent. These differences highlight the suitability of HHP as an innovative extraction technique to recover a greater amount of phenolic compounds from watercress using shorter processing times and greener solvents.

3.4. Clustering of phenolic compounds according to the HHP conditions that maximize their extraction

Table 4 shows the maximum response values of each phenolic compound and their values if extracted under the optimal HHP

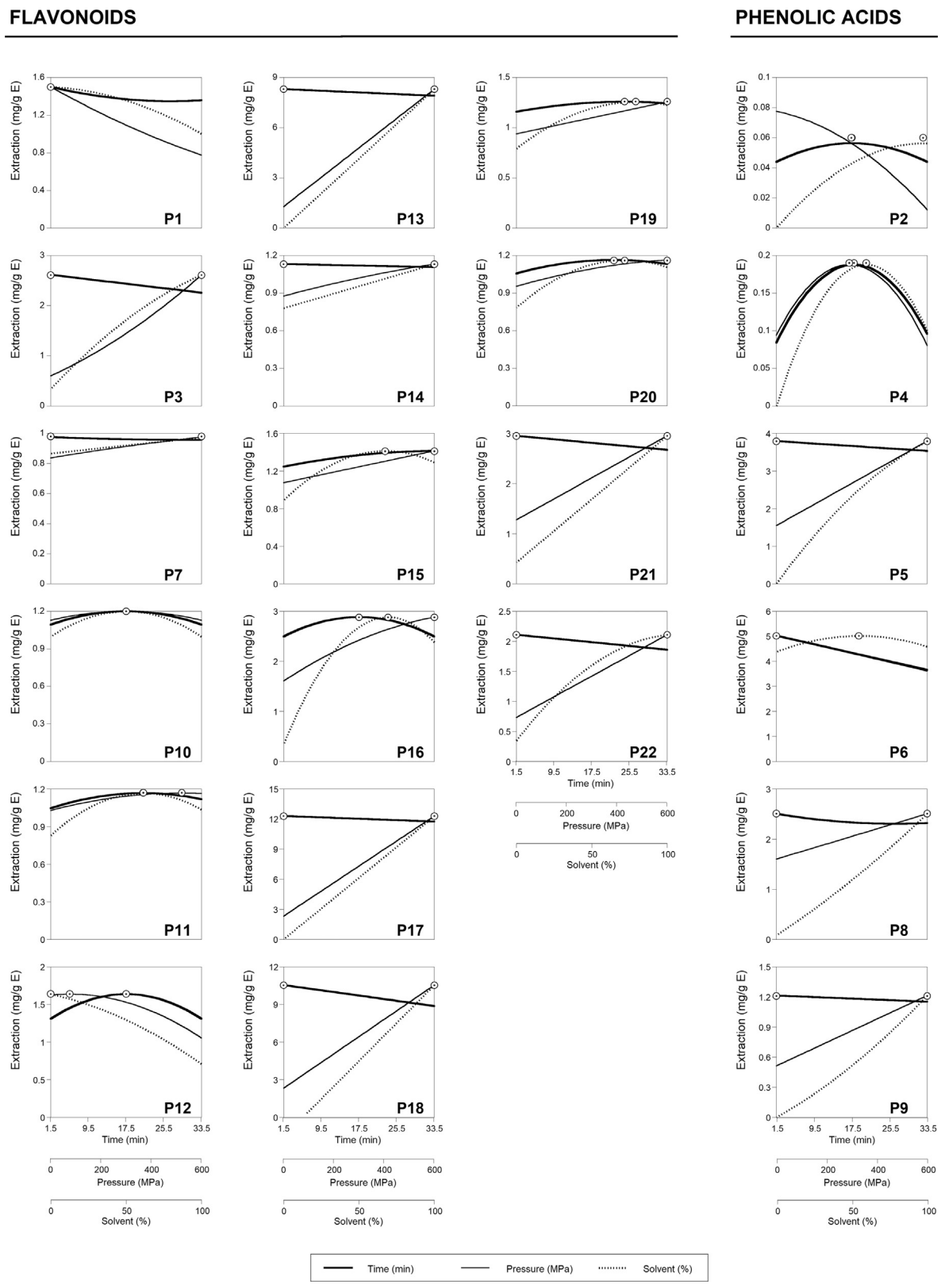

Fig. 4. 2D graphical response of the effects of the independent variables on the extraction of phenolic compounds from watercress (see Table 1 for peak identification). Dots (⊙) represent the optimal values. In each plot, each independent variable was positioned at the optimal value of the other two variables (Table 3).

conditions of the other compounds (Table 3). These values presented in part B were calculated dividing the optimum value of each compound by the maximum of the others compounds. Therefore, when two compounds display the value 1 (corresponding to values of 100%), the optimum response value of both compounds is achieved under the same HHP conditions. This is the case of compounds 3, 5, 7, 8, 9, 13, 14, 17, 18, 21 and 22, which were clustered in C3a under the same HHP conditions (Fig. 5). In turn, when a 0 is display, it means that the

Table 4
Maximum response values for each phenolic compound and their values at the optimal processing conditions of the other compounds presented in Table 2.

A) Maximum response values (mg/g of extract) for the individual phenolic compounds

Peak:	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22
Optimum:	1.50	0.06	2.61	0.19	3.79	5.02	0.98	2.51	1.21	1.20	1.17	1.64	8.31	1.13	1.41	2.88	12.30	10.54	1.26	1.16	2.95	2.11

B) Values for each phenolic compound (%) at the optimal conditions of the other compounds

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22
P1	*1*	0.69	0.85	0.75	0.85	0.89	0.85	0.85	0.85	0.76	0.79	0.85	0.85	0.85	0.90	0.81	0.85	0.85	0.86	0.84	0.85	0.85
P2	0	*1*	0	0.86	0	0.59	0	0	0	0.76	0.54	0	0	0	0.16	0.38	0	0	0.30	0.35	0	0
P3	0.90	0.49	*1*	0.57	1	0.62	1	1	1	0.58	0.66	0.81	1	1	0.73	0.77	1	1	0.81	0.78	1	1
P4	0	0.59	0	*1*	0	0.05	0	0	0	0.97	0.67	0	0	0	0.01	0.35	0	0	0.19	0.33	0	0
P5	0.82	0.62	1	0.67	*1*	0.71	1	1	1	0.67	0.74	0.77	1	1	0.82	0.81	1	1	0.87	0.83	1	1
P6	0.87	0.91	1	0.87	1	*1*	1	1	1	0.84	0.90	0.76	1	1	1	0.95	1	1	1	1	1	1
P7	0.97	0.94	1	0.93	1	0.91	*1*	1	1	0.92	0.92	0.95	1	1	0.92	0.94	1	1	0.95	0.94	1	1
P8	0.55	0.69	1	0.52	1	0.56	1	*1*	1	0.48	0.57	0.50	1	1	0.67	0.64	1	1	0.74	0.67	1	1
P9	0.35	0.66	1	0.41	1	0.32	1	1	*1*	0.37	0.51	0.33	1	1	0.59	0.62	1	1	0.72	0.64	1	1
P10	0.68	0.85	0.68	0.99	0.68	0.85	0.68	0.68	0.68	*1*	0.95	0.80	0.68	0.68	0.83	0.91	0.68	0.68	0.87	0.90	0.68	0.68
P11	0.73	0.85	0.78	0.98	0.78	0.88	0.78	0.78	0.78	0.98	*1*	0.76	0.78	0.78	0.96	0.99	0.78	0.78	0.98	0.99	0.78	0.78
P12	0.79	0.70	0.63	0.86	0.63	0.50	0.63	0.63	0.63	0.89	0.85	*1*	0.63	0.63	0.63	0.84	0.63	0.63	0.80	0.82	0.63	0.63
P13	0.39	0.54	1	0.39	1	0.26	1	1	1	0.36	0.54	0.31	*1*	1	0.63	0.67	1	1	0.76	0.69	1	1
P14	0.85	0.89	1	0.84	1	0.81	1	1	1	0.83	0.86	0.83	1	*1*	0.88	0.89	1	1	0.92	0.90	1	1
P15	0.77	0.77	0.79	0.92	0.79	0.92	0.79	0.79	0.79	0.92	0.96	0.72	0.79	0.79	*1*	0.97	0.79	0.79	0.98	0.98	0.79	0.79
P16	0.24	0.55	0.69	0.87	0.69	0.53	0.69	0.69	0.69	0.87	0.96	0.37	0.69	0.69	0.87	*1*	0.69	0.69	0.96	0.99	0.69	0.69
P17	0.35	0.56	1	0.40	1	0.26	1	1	1	0.36	0.54	0.28	1	1	0.63	0.67	*1*	1	0.76	0.69	1	1
P18	0.23	0.52	1	0.36	1	0.23	1	1	1	0.33	0.49	0.26	1	1	0.57	0.62	1	*1*	0.69	0.63	1	1
P19	0.78	0.82	0.90	0.90	0.90	0.85	0.90	0.90	0.90	0.90	0.96	0.77	0.90	0.90	0.98	0.99	0.90	0.90	*1*	1	0.90	0.90
P20	0.76	0.85	0.86	0.96	0.86	0.86	0.86	0.86	0.86	0.96	0.99	0.78	0.86	0.86	0.97	0.99	0.86	0.86	1	*1*	0.86	0.86
P21	0.45	0.66	1	0.53	1	0.44	1	1	1	0.51	0.63	0.46	1	1	0.69	0.72	1	1	0.78	0.73	*1*	1
P22	0.51	0.63	1	0.69	1	0.60	1	1	1	0.68	0.79	0.52	1	1	0.84	0.87	1	1	0.89	0.88	1	*1*

conditions that maximize the extraction of a certain compound (compounds 1, 3, 0, 7, 8, 9, 12, 13, 14, 17, 18, 21 and 22) do not favour at all the extraction of the other one (compounds 2 and 4).

Using the complete dataset of Table 4 and performing a multi-objective optimization problem using an appropriate clustering algorithm, different clusters of phenolic compounds whose maximum response values are obtained under similar HHP extraction conditions were created. The results of HCA are presented in Fig. 5. In the HCA dendrogram, the shorter distance between compounds indicates a higher similarity in terms of conditions that favour their extraction and the compounds clustered into the same group are better extracted under similar HHP conditions. Three significant clusters (C1, C2 and C3) were generated. C1 and C3 were also be divided in two (a and b) pertinent subgroups. Additionally, other less relevant subgroups were created in C2, C1b and C3b, but they can be considered as a residual noise produced by the algorithm.

– Cluster C1 included the compounds 15, 16, 11 and 10. Meanwhile, compound 15 was subdivided in C1a and compounds 16, 11 and 10 were grouped in C1b. The extraction of these compounds was maximized by medium *t*, high *P* and medium *S* (Table 3 and Fig. 3). The subgroups C1a and C1b were mainly differentiated by the *t* values.
– Cluster C2 included the compounds 4, 20 and 19. No significant subgroups were created. The extraction of these compounds was favoured by medium *t*, high *P* and medium-large *S* values.
– Cluster C3 included the compounds 22, 21, 18, 17, 14, 13, 9, 8, 7, 3, 5, 12, 6, 1 and 2, which were subdivided in C3a e C3b. The extraction of the compounds in C3a was maximized when using low *t*, high *P* and high *S*. On the other hand, the compounds in C3b exhibited a broad set of conditions with no clear interconnections between each other.

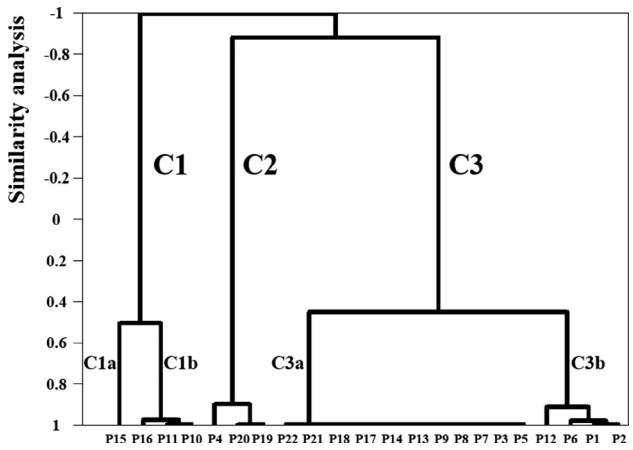

Fig. 5. Results of the hierarchical cluster analysis of phenolic compounds according to the HHP conditions that maximize their extraction from watercress.

Although it was expected that compounds with similar chemical characteristics would exhibit comparable optimal extraction conditions, no clear similarity was detected between the created groups of compounds and the conditions that maximize their extraction. However, this HCA analysis was an interesting and innovative approach in the field of extraction of high added-value compound from natural sources (something not seen in this type of studies), since it allowed grouping the phenolic compounds into different clusters according to the HHP conditions that favour their recovery from watercress, which can be very useful from a practical point of view.

4. Conclusions

As far as we know, this is the first study regarding the optimization of the extraction of phenolic compounds from watercress by HHP using RSM, a suitable statistical tool that allowed reduce the number of experimental trials and evaluate interactions among variables. The suitability of this cold extraction method (combining the independent variables *t*, *P* and *S* in a five-level CCCD design) was demonstrated. The developed polynomial response models were statistically validated and expressed as 2D and 3D surface plots to better visualize the effects on extraction yield and individual and grouped phenolic compounds (a total of 29 response variables). A good agreement between

experimental and theoretical results was observed. In general, the recovery of phenolic compounds was maximized when high pressures, high ethanol concentrations and short extraction times were applied, which validate this cold extraction method as a very promising technique compared to the time-consuming conventional methods. This study also highlighted watercress as being an interesting source of phytochemicals, namely phenolic acids and flavonoids.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT) of Portugal and FEDER under Programme PT2020 for financial support to CIMO (UID/AGR/00690/2013); to FCT/MEC for financial support to REQUIMTE/LAQV (UID/QUI/50006/2013 - POCI/01/0145/FERDER/007265) through national funds and co-financed by FEDER under Programme PT2020; to FCT/MEC for the financial support to the QOPNA research Unit (FCT/ID/QUI/00062/2013), through national funds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement; to FCT for the grant attributed to J. Pinela (SFRH/BD/92994/2013) through the Programa Operacional Capital Humano (POCH) supported by the European Social Fund and National Funds of MCTES (Ministério da Ciência, Tecnologia, e Ensino Superior); to FCT for L. Barros research contract; and to the Xunta de Galicia for financial support to M.A. Prieto. The authors are also grateful to the Interreg España-Portugal for financial support through the project 0377_Iberphenol_6_E.

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