



Design of experiments (DoE) to model phenolic compounds recovery from grape pomace using ultrasounds

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Revised: 9 October 2021 / Accepted: 4 November 2021 / Published online: 22 November 2021
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Abstract Grape processing by-products (particularly grape pomace) are known to contain high amounts of phenolic compounds. To improve the extraction of phenols from this by-product, it is necessary to develop a method and set and model optimal conditions for their extraction. By applying the design of experiments (DoE) approach, optimal experimental factors of Ultrasound-assisted extraction (USAE) were determined to obtain grape pomace extracts with a satisfactory yield of phenols anthocyanins, as well as extracts with high antioxidant capacity using reagents approved in the food industry. Initial method optimization covered two experimental factors: solvent concentration and the weight ratio of the sample and solvent using fixed USAE conditions from literature. For the final method optimization, the three

investigated experimental factors were: pH value, the temperature of extraction, and extraction time. The optimal experimental conditions for the development of the method were 55% ethanol, sample/solvent ratio 1:40, pH 4.5, T 55 °C, and 30 min. Depending on the primary goal of the extraction process (the antioxidant activity, total phenolic content, content of individual phenols, or content of individual anthocyanins), these parameters can easily be modified to obtain the desired recovery.

Keywords Grape pomace · By-product · Phenolic compounds · Ultrasound-assisted extraction · Optimization

Abbreviations

DoE Design of experiments
USAE Ultrasound-assisted extraction

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Introduction

Grape species *Vitis vinifera* is one of the most cultivated fruit crops globally, with an annual production of more than 67 million tons (Kammerer et al. 2005). Grapes have typically crushed a product (must) and a by-product (pomace), which consists of seeds, skins, and stems, and typically contains 30–40% of phenolic compounds found in grapes (Georgiev et al. 2014). The most common grape pomace phenols are anthocyanins, hydroxybenzoic and hydroxycinnamic acids, flavan-3-ols, flavonols, stilbenes, monomeric and oligomeric proanthocyanidins. However, their composition and presence in the grape's pomace vary significantly concerning the different factors, such as the variety and vintage of grapes and the applied technological process during fermentation. Various in vitro and in vivo studies have shown that the substances present in grape pomace and must exhibit antioxidant, anti-inflammatory, cardioprotective, vasoprotective, anti-cancer, anti-diabetic, and antimicrobial activity (Cvejic et al. 2017). Besides, food ingredients are today investigated as bioactive compounds that support the immune system against COVID-19 disease (Galanakis, 2020). For instance, polyphenols and flavonoids like protocatechuic acid, kaempferol, and epigallocatechin gallate have been proposed as potential inhibitors of the main SARS-CoV-2 protease in molecular docking studies (Galanakis et al. 2020).

Therefore, grape by-products can be used in the food industry and cosmetics, as a raw material for producing phenol-rich dietary supplements and phytochemicals and in oenological applications (Cvejić Hogervorst et al. 2017; Galanakis 2018; Galanakis et al. 2018). The recovery of high added-value compounds from food processing by-products such as grape pomace is known to be accomplished following the so-called “5-Stages Universal Recovery Process”. The latest approach is based on the following steps: (I) macroscopic pretreatment, (II) separation of macro- and micro-molecules, (III) extraction, (IV) purification, and (V) product formation (Galanakis 2012, 2015). The third step, extraction (particularly liquid–solid), is the most crucial stage and can be described as a mass transport phenomenon in which insoluble solids (contained in grape pomace) migrate into a solvent that comes into contact with the matrix (Galanakis et al. 2013). The main demerit of traditional methods, such as solid–liquid or Soxhlet extraction, is that they require relatively large amounts of solvents, high temperatures, and are long-lasting (Fontana et al. 2013). Mass transport phenomena can be enhanced by changes in concentration gradients, diffusion, coefficients, or boundary layer, whereas ultrasonics' cavitations and disrupting properties have been used for this purpose (Barba et al. 2015).

Ultrasonic radiation can significantly increase the extraction efficiency of using a single extraction step. It is simple, inexpensive, and represents a minor modification of conventional solid–liquid extraction with a significant advantage to reduce extraction time without the need for high temperatures that can affect the stability of phenols (Fontana et al. 2013; Ghafoor et al. 2009). Ultrasounds have been used for the enhanced recovery of anthocyanins and β -carotene extraction from grape seeds, citrus, and pomegranate peels (Ghafoor et al. 2009; Pan et al. 2012). For instance, continuous ultrasound-assisted extraction of polyphenols from pomegranate peels increased the antioxidant yield by 24% compared with conventional liquid extraction and reduced the extraction time by 90% (Pan et al. 2012).

On the other hand, the industrial implementation of non-thermal technologies like ultrasounds is affected by the equipment and general cost. Therefore, they could be too sophisticated compared to the yield improvement that they are promising (Galanakis 2015). Besides, one of the difficulties reported in the literature is the lack of non-standardized methodologies and the optimization of control parameters (Galanakis 2013). For example, Heng et al. (2015) used a two-level factorial design to optimize flavonoids' recovery from palm kernel by-products. Ghafoor et al. (2009) applied response surface method (RSM) to optimize maximize the extraction of phenols from grape seed using ultrasonic radiation. The studied parameters were the total phenolic content, total anthocyanins, and antioxidant activity; the optimized conditions were shown to be an ethanol concentration of about 53%, at a temperature of 55–60 °C, and at an extraction time of about 30 min (Ghafoor et al. 2009). However, the pH value of the extraction medium and the weight ratio of sample and solvent, which are critical conditions of the extraction process, was not optimized. Roselló-Soto et al. (2015) revised the modeling approaches to recover antioxidant compounds from plant processing by-products using ultrasounds and found design of experiments (DoE) one of the most reliable statistical methods.

This work aims to determine optimal experimental conditions of Ultrasound-assisted extraction (USAE) to obtain grape pomace extracts with a satisfactory yield of phenols, including anthocyanins, and extracts with high antioxidant capacity. To use obtained extracts in the food industry, extraction of phenols by ethanol and water was applied. Citric acid and sodium citrate, which are also approved in the food industry, were used to correct the extraction medium's pH value. The DoE approach performed the optimization, which allows for the prediction of optimal values for experimental factors from previously planned experiments with the help of mathematical criteria included in the factorial design.

Material and methods

Chemicals and reagents

All standards of phenolic compounds were purchased from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland), or ChromaDex (Santa Ana, USA). Anthocyanins, malvidin 3-*O*-glucoside (oenin chloride), cyanin 3-*O*-glucoside chloride (kuromanin chloride), and delphinidin 3-*O*-glucoside chloride (delphinidin, myrtillin) obtained from AppliChem, petunidin 3-*O*-glucoside chloride, and peonidin 3-*O*-glucoside chloride from Phytolab. Methanol and acetonitrile HPLC grade were from Promochem LGC (Wesel, Germany), formic acid from Lach-Ner (Neratovice, Czech Republic), ethanol 96% from Zorka Pharma (Šabac, Serbia), acetic acid 99% from Fluka Chemie GmbH (Buchs, Switzerland) and hydrochloric acid 35%, citric acid and sodium citrate from Roth (Karlsruhe, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) purchased from Sigma-Aldrich Chem (Steinheim, Germany) while Folin-Ciocalteu reagent from Fluka Chemie GmbH (Buchs, Switzerland).

Sample preparation

The extraction of phenols included pomace obtained after removal from pressed red grapes of *Merlot* variety from Krčedin, Serbia. After drying grape pomace at room temperature (25 °C) for three days, the next step was grinding in a blender to a powder consistency.

Extraction procedure

After sample preparation, the proper pomace mass was weighed on an analytical balance to set the solvent and pomace mass ratio. Pomaces were quantitatively transferred to the volumetric flasks of 10 mL, which were filled with an appropriate extraction medium. After that, the volumetric flasks were placed in an ultrasonic bath (Bandelin Sonorex, Berlin, Germany) and held at the proper time and temperature (as enclosed in Tables 1 and 2, respectively). The model of the ultrasonic bath was JYD-1012SG with ultrasonic power of 600 W, while the ultrasonic frequency was 28 kHz. After being cooled, the extracts were filtered through a membrane filter (0.45 μm) and analyzed.

Experimental design

MODDE 10.0 Software (Umetrics, Umea, Sweden) was used to optimize experimental factors through an appropriate factorial design. Through the set of 11 planned

experiments (Plackett Burman design), the two experimental factors initially investigated were: solvent concentration (x_1) ranged from 0% (i.e., 100% water) to 80% ethanol in water and the weight ratio of sample and solvent (x_2) ranged from 1:10 (i.e., 1 g sample per 10 mL solvent) to 1:60 (i.e., 1 g sample per 60 mL solvent), using fixed US extraction conditions from literature: 55 °C, 30 min (Table 1). The ranges were defined based on scientific background, practical and financial implications regarding the extraction process. For example, previous works showed that the maximum ethanol concentration in water used to extract phenols from grape pomace was 80%, and the ratio of the sample and solvent 1:60 (Fontana et al., 2013).

For the final method optimization, a set of 17 planned experiments within the CCF (2^3 full factorial Central Composite Face) design was applied (Table 2). The three experimental factors investigated were: pH value (x_1) from 3 to 6, the temperature of extraction (x_2) from 30 to 60 °C and time of extraction (x_3) from 10 to 60 min using solvent concentration and the weight ratio of sample and solvent that gave the best results from initial optimization. Data from literature demonstrated that the phenolic extract obtained at pH 4.5 had a higher proportion of total and individual phenols and better antioxidant capacity than the extract obtained at a lower pH value (Rubio-Senent et al. 2017). The pH mentioned above (4.5) is set to be the middle value of the range. As citric acid is a weak organic acid and can efficiently maintain a pH from 3 to 6.2, we determined our range from pH 3–6 (Dawson et al. 1986). Higher temperatures damage phenolic compounds and cause a loss in the yield (Spigno et al. 2007), so we set the maximum temperature at 60 °C to prevent phenolic degradation. The extraction time is limited to 60 min due to practical reasons and the whole cost of the extraction process.

The significance of the factors assessed by calculating the factorial coefficients (b) of the corresponding polynomial given in Eq. (1) for the Plackett Burman design and (2) for the full factorial CCF DoE:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + \dots + b_Nx_N \quad (1)$$

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + \dots + b_Nx_N + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + \dots + b_{(N-1)N}x_{N-1}x_N + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + \dots + b_{NN}x_N^2 \quad (2)$$

where x is the experimental factor, y represents the estimated response, b_0 is the average experimental response, coefficients b_N are the estimated effects of the factors, the coefficients $b_{(N-1)N}$ are the interaction terms and the b_{NN} are second-order terms.

Table 1 Assessment of the antioxidant activity (y_1) and total phenolic content (y_2) during the initial screening for extraction conditions using Plackett Burman design of experiments; one sample under different experimental conditions: ethanol concentration (x_1) and sample/solvent ratio (x_2)

Exp. No	Experimental factors		Extraction efficacy descriptors ^a	
	x_1 [c(ethanol), %]	x_2 (sample/solvent ratio, w/V)	y_1 Antioxidant activity (IC_{50} , $\mu\text{L}/\text{mL}$)	y_2 Total phenolic content (TPC, mg GAE/L)
No. 1	80	10	0.55 ± 0.04	2280 ± 36
No. 2	80	60	0.51 ± 0.05	2542 ± 19
No. 3	80	60	0.41 ± 0.04	2661 ± 23
No. 4	0	60	1.80 ± 0.14	910 ± 11
No. 5	80	10	0.59 ± 0.12	2302 ± 16
No. 6	0	60	1.76 ± 0.22	908 ± 28
No. 7	0	10	3.16 ± 0.28	809 ± 12
No. 8	0	10	3.35 ± 0.30	806 ± 17
No. 9	40	35	0.44 ± 0.07	2686 ± 37
No. 10	40	35	0.43 ± 0.07	2702 ± 42
No. 11	40	35	0.42 ± 0.05	2799 ± 56

^aResults are the means ± SD ($n = 3$)

Table 2 Assessment of the antioxidant activity (y_1) and total phenolic content (y_2) during the optimization of the extraction conditions using 2^3 full factorial Central Composite Face design of experiments; under different experimental conditions: pH value (x_1), temperature of extraction (x_2) and time of extraction (x_3)

Exp. No	Experimental factors			Extraction efficacy descriptors ^a	
	x_1 pH value	x_2 Temperature ($^{\circ}\text{C}$)	x_3 Time (min)	y_1 Antioxidant activity (IC_{50} , $\mu\text{L}/\text{mL}$)	y_2 Total phenolic content (TPC, mg GAE/L)
No. 1	3	30	10	0.91 ± 0.01	1841 ± 35
No. 2	6	30	10	0.64 ± 0.05	2253 ± 41
No. 3	3	60	10	0.48 ± 0.06	3077 ± 32
No. 4	6	60	10	0.35 ± 0.03	2177 ± 16
No. 5	3	30	60	0.76 ± 0.05	2842 ± 17
No. 6	6	30	60	0.62 ± 0.07	2512 ± 24
No. 7	3	60	60	0.52 ± 0.58	3236 ± 52
No. 8	6	60	60	0.31 ± 0.04	2300 ± 14
No. 9	3	45	35	0.45 ± 0.06	3006 ± 18
No. 10	6	45	35	0.32 ± 0.05	2400 ± 11
No. 11	4.5	30	35	0.50 ± 0.09	2518 ± 27
No. 12	4.5	60	35	0.36 ± 0.03	3148 ± 33
No. 13	4.5	45	10	0.39 ± 0.06	3412 ± 31
No. 14	4.5	45	60	0.37 ± 0.05	3706 ± 48
No. 15	4.5	45	35	0.36 ± 0.03	3736 ± 26
No. 16	4.5	45	35	0.35 ± 0.02	3677 ± 29
No. 17	4.5	45	35	0.34 ± 0.03	3677 ± 61

^aResults are the means ± SD ($n = 3$)

Determination of the antioxidant activity

The antioxidant activity was determined spectrophotometrically by reaction with DPPH (2,2-diphenyl-1-picryl-

hydrazyl) radical (Brand-Williams et al. 1995). An appropriate growing amount of grape pomace extract (50–250 μL), 1000 μL of ethanolic solution of DPPH reagent (90 μM), and ethanol to 4000 μL were added and

represented sample. The control sample was prepared by adding 1000 μL of the working DPPH solution to 3000 μL of ethanol. After leaving it for 1 h in the dark and at room temperature, its absorbance was read on a spectrophotometer (Jenway 6405 UV/Vis, Essex, UK) at a wavelength of 515 nm, with a 95% ethanol solution as a reference. All tests were carried out in triplicate. Radical scavenging capacity (RSC) was calculated for each concentration according to the equation $\text{RSC} = 100 - (100 \times [A_{\text{sample}}/A_{\text{control}}])$, where A_{sample} represented absorbance of the analyzed samples, and A_{control} represented absorbance of the control. A calibration curve was constructed so that RSC is against the concentration of grape pomace extracts. A 50% inhibitory concentration (IC_{50} , $\mu\text{L}/\text{mL}$) was calculated as a concentration of extract necessary for achieving RSC in the amount of 50%.

Determination of total phenolic content

The content of total phenolic compounds in grape pomace extracts was determined by the Folin–Ciocalteu's (FC) method using gallic acid as standard (Hagerman et al. 2000). Before analysis, all grape pomace extracts were filtered through a membrane filter (0.45 μm) and diluted ten times with distilled water. The absorbance of the standards and samples was measured at 740 nm after 2 h in the dark at room temperature. All tests were carried out in triplicate. The results were expressed as mg of gallic acid equivalents (mg GAE/L).

Determination of individual phenols

The determination was performed following conditions previously stated by Miljić et al. (2017): direct separation of phenols in 5 μL of the sample by HPLC using a reverse-phase Poroshell 120 EC-C18 column (4.6 \times 100 mm, 2.7 μm ; Agilent, USA) at 25 $^{\circ}\text{C}$ with gradient elution by a mixture of 0.1% acetic acid in water (A) and acetonitrile (B) (B: 0 min 8%; 3.25 min 10%; 8 min 12%; 15 min 25%; 15.8 min 30%; 24 min 50%, 25 min 100%, 27 min 100%, 28 min 8%; flow rate 1 mL/min) and with detection at the following wavelengths (nm): 225 (vanillic acid, benzoic acid), 280 (gallic acid, 4-hydroxy benzoic acid, catechin, syringic acid, *trans*-cinnamic acid, hesperetin, naringenin), 305 (*p*-coumaric acid), 330 (chlorogenic acid, caffeic acid, resveratrol), and 360 (rutin, quercetin, kaempferol). Quantification of the compounds of interest was performed using a calibration curve obtained by using calibration solutions. All tests were carried out in triplicate.

Determination of anthocyanins

Stock solutions of individual anthocyanins (malvidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, delphinidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and peonidin 3-*O*-glucoside) were prepared in methanol acidified with HCl to 1%. Calibration standards were prepared as mixtures of all the five components diluted with the initial mobile phase, in the concentration range 1–100 mg/L (r^2 above 0.998 for all anthocyanins). Samples were prepared by filtration through a membrane filter (0.45 μm ; Sartorius, USA). HPLC analysis was carried out using Agilent 1100 series liquid chromatography (USA), consisting of a quaternary gradient pump, autosampler with injection system (10–200 μL), column heater, UV–VIS detector, and software package. Chromatographic separation of five major anthocyanins in 100 μL of the sample was achieved on a reverse-phase Poroshell 120 EC-C18 column (4.6 \times 100 mm, 2.7 μm ; Agilent, USA) heated at 40 $^{\circ}\text{C}$ with gradient elution by water/formic acid/acetonitrile mixtures (A—87:10:3, B—40:10:50; gradient: 0 min 6% B, 9 min 30% B, 10 min 30% B, 11 min 60% B, 14 min 60% B; flow rate 0.8 mL/min; run time 14 min) and followed by detection at 518 nm [method based on Compendium of International Methods of Analysis—OIV (Beara et al. 2017)]. All tests were carried out in triplicate. Quantification of the compounds of interest was performed using the calibration curves obtained from injections of calibration solutions. Sensitivity of the determination was achieved with the increase of injected volume of the samples, four folds higher than the standard mixture, thus enabling quantification of less abundant compounds.

Results and discussion

Initial screening for critical factors affecting extraction and their effect on defined extraction efficacy descriptors

The results obtained during the initial screening to detect the most significant factors affecting extraction are enclosed in Table 1 and Fig. 1. The results showed that both factors (solvent concentration and sample to solvent ratio) affect the extraction efficacy. Still, the direction of the impact of both factors varied among each of the defined extraction responses.

According to contour diagrams of the extraction efficacy descriptors over the defined area of the experimental factors, better antioxidant activity and total phenolic content

of the extracts (lower IC_{50} and higher TPC values) were achieved concentration of ethanol. The influence of the sample/solvent ratio was not crucial for this response. Most of the developed extraction methods aim to obtain extracts with a high polyphenols content instead of fractionation and extraction of one or more related substances. Polyphenols are easily solubilized in polar protic mediums such as hydroalcoholic mixtures (Galanakis et al. 2013). Therefore, several researchers have used organic solvent/alcohol/water solvent systems to extract compounds from grape processing by-products. The presence of water increased showed a satisfactory yield of both anthocyanins and phenols of molecules by diffusion, leading to higher output of water-soluble substances (Cheng et al. 2012; Jayaprakasha et al. 2001). In terms of extracting total phenolic content, ethanol/water mixtures gave better results than acetone or methanol/water solvent system (Fontana et al. 2013). The preference of polyphenols between methanol and ethanol can be attributed to their nonpolar part and the aliphatic fragment of alcohols. Methanol contains a smaller and more flexible aliphatic component than ethanol and thus surrounds easier polyphenols with 3 or 4 substituted carbons inside their aromatic ring (e.g., vanillic and syringic acids). On the other hand, larger molecules, polyphenols with two anti-diametric substituted carbons or longer aliphatic fragments prefer ethanol

(Galanakis et al. 2013). Nevertheless, ethanol is a less expensive and safer solvent that can be used in the food industry (Fontana et al. 2013).

Using the same experimental conditions as described with the Plackett Burman design of experiments (Table 1), the content of individual phenols and anthocyanins was determined (chromatograms of standards and selected samples of phenols and anthocyanins are provided via Supplementary data). The effect of the extraction efficacy descriptors: individual content of phenols (kaempferol, catechin, vanillic acid, syringic acid, gallic acid, *trans*-cinnamic acid, naringenin, hesperetin, rutin, and quercetin) and anthocyanins (delphinidin-3-gl, cyanidin-3-gl, petunidin-3-gl, peonidin-3-gl, malvidin-3-gl) over the defined area of the experimental factors solvent concentration (x_1) ranged from 0% (i.e., 100% water) to 80% ethanol in water and the weight ratio of sample and solvent (x_2) ranged from 1:10 to 1:60 (using fixed US extraction conditions: 55 °C, 30 min) was assessed using RSM (Fig. 2).

The defined experimental factors had variable effects on the extraction of phenolic compounds; except for gallic acid, better extraction of phenolic compounds was achieved when higher ethanol concentrations were used. Besides, it is known that gallic acid (where three hydroxyl groups and one carboxyl group are surrounding the aromatic ring) is preferably solubilized in a polar protic water

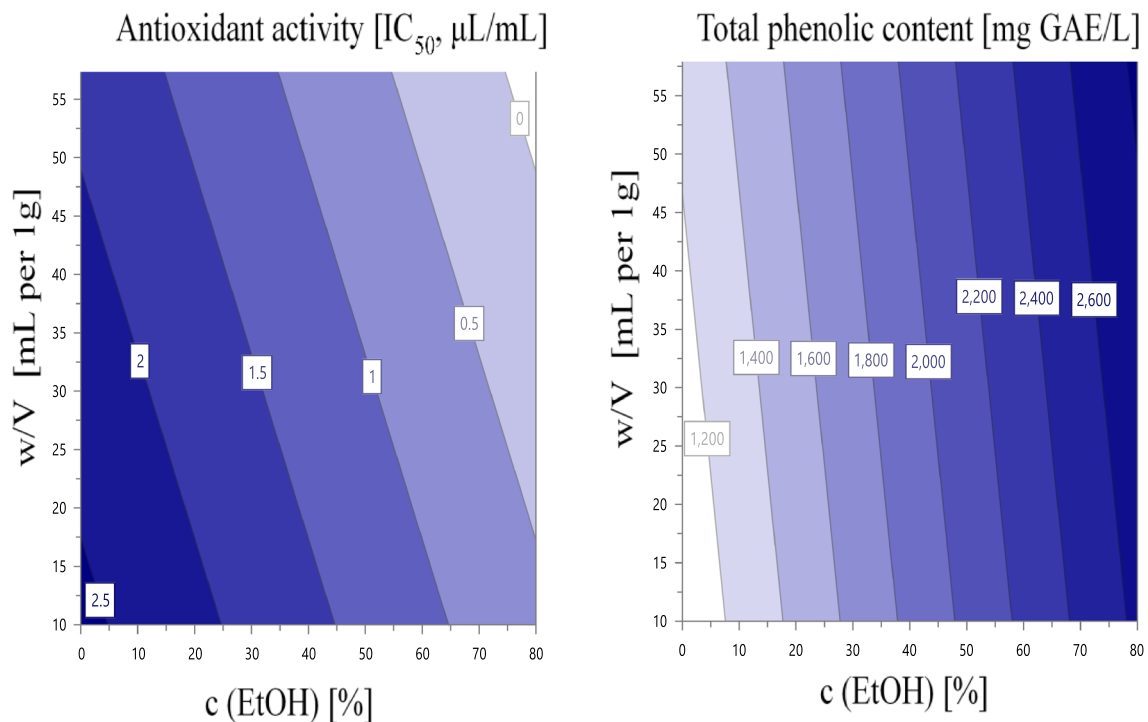


Fig. 1 Contour diagram of the influence of the change of the solvent concentration (x_1) ranged from 0% (i.e. 100% water) to 80% ethanol in water and weight ratio of sample and solvent (x_2) ranged from 1:10 to 1:60 (using fixed US extraction conditions: 55 °C, 30 min) on the

antioxidant activity (y_1) and total phenolic content (y_2) during the initial screening for extraction conditions using Plackett Burman design of experiments

molecule (Galanakis et al. 2013). To extract two phenols of interest (vanillic acid, syringic acid), ethanol concentration did not affect. Here, better results were achieved using a smaller sample/solvent ratio (1 g sample: 10 mL solvent). A similar conclusion was obtained for naringenin and hesperetin, but for other phenols (kaempferol, rutin, quercetin); it was quite the opposite, i.e., better extraction achieved when the sample/solvent ratio was more significant (1 g sample: 60 mL solvent). And finally, there were phenols (catechin, gallic acid, *trans*-cinnamic acid) for which the sample/solvent ratio had no impact. Thereby using DoE to optimize the extraction efficacy revealed that solvent/sample ratio was a critical factor to be assessed, and its effect on the extraction efficacy is more apparent if the optimization was made traditionally.

The influence of ethanol concentration on the extraction yield of anthocyanins was the opposite of the antioxidant activity results. The majority of the phenols (Fig. 2), i.e., better recovery, were achieved using a smaller concentration of ethanol in water, concluding that pure water is the best extraction solvent for all anthocyanins of interest.

Aiming toward an extraction method suitable for extracting anthocyanins simultaneously with phenolic compounds, the concentration of ethanol was set to 55%. This is the area where each contour diagram showed a satisfactory yield of both anthocyanins and phenols. Additionally, approximate concentration (50%, 53%, and 60% ethanol, respectively) is commonly used in literature for similar purposes, so the obtained results in this research would be comparable with the results alike (Vatai et al. 2009; Ghafoor et al. 2009; Amendola et al. 2010).

For the sample/solvent ratio, it can be concluded that better extraction of anthocyanins was achieved when the sample/solvent ratio was more significant (1 g sample: 60 mL solvent). Having in mind the variable effect of the sample/solvent rate over-extraction of phenols, the opposite results obtained for the extraction of most phenols and anthocyanins, as well as the impact on antioxidant activity and total phenolic content from the aspect of this factor, was concluded to choose the value for this factor based on the results obtained by the RSM (Response Surface methodology) (Ghafoor et al. 2009). It was proposed that the optimal sample/solvent ratio for this research would be the central value of the experimental range (1:40, 1 g sample: 40 mL solvent) as this value covers satisfactory results for all the responses when the concentration of ethanol was set on 55%.

Optimization of extraction parameters

This part of the research aimed at further optimization of the antioxidant activity, extraction of anthocyanins (delphinidin-3-gl, cyanidin-3-gl, petunidin-3-gl, peonidin-3-gl,

and malvidin-3-gl), and phenols (kaempferol, catechin, vanillic acid, syringic acid, gallic acid, *trans*-cinnamic acid, naringenin, hesperetin, rutin, and quercetin), whereas the extraction time would be reasonably low. A set of 17 planned experiments within the CCF (2^3 full factorial Central Composite Face) design applied during the final method optimization comprised investigation of three experimental factors: pH value (x_1) from 3 to 6, the temperature of extraction (x_2) from 30 to 60 °C and time of extraction (x_3) from 10 to 60 min (Table 2). The effects of the different experimental conditions on the defined extraction yield descriptors (antioxidant activity, extraction of phenols, and anthocyanins extraction) are shown through the normalized DoE coefficients (Fig. 3A–C, respectively).

The DoE models were fitted for their intended use with MLR (Multiple Linear Regression), and the intended use of the experiments was verified. The determination coefficients (R^2) for all extraction yield descriptors above 0.72. A lack-of-fit error was estimated by included replicate experiments at a central point, which proved satisfactory reproducibility of the models (variation of the replicates compared to overall variability above 0.87). Residuals standard probability plots showed normally distributed noise. Additionally, the expected yield of anthocyanins and phenols, along with the satisfactory antioxidative activity, was cross-verified by selected experiments at the defined optimum condition for desired analytes that yielded expected results.

Normalized coefficients of the 2^3 CCF full factorial DoE showed that extraction time (factor x_3) has an almost negligible effect on the extraction efficacy, i.e., this factor influenced only the catechin extraction, gallic acid. And kaempferol (positively for the first two, and simultaneously opposite for the latter). For this reason, and to evaluate the influence of the other two examined parameters (pH value and temperature) on the antioxidant activity, total phenolic content, the content of anthocyanins, and content of phenols, the extraction time was set at the median value of the examined interval (30 min) (Fig. 4).

According to contour diagrams of the extraction efficacy descriptors over the defined area of the experimental factors, optimal antioxidant activity and total phenolic content of the extracts were achieved at higher temperature and pH values between 4 and 5. The temperature increases extraction efficiency by improving the solubility of the solute and diffusion coefficient. However, at a temperature higher than 50 °C, polyphenols' stability is reduced, resulting in their denaturation. Besides, polyphenols are reactive chemical species, vulnerable to oxidation, conjugation, hydrolysis, polymerization, and complexation. Similarly, similar results have been found in other studies that referred to that heating at 50 or 60 °C caused a

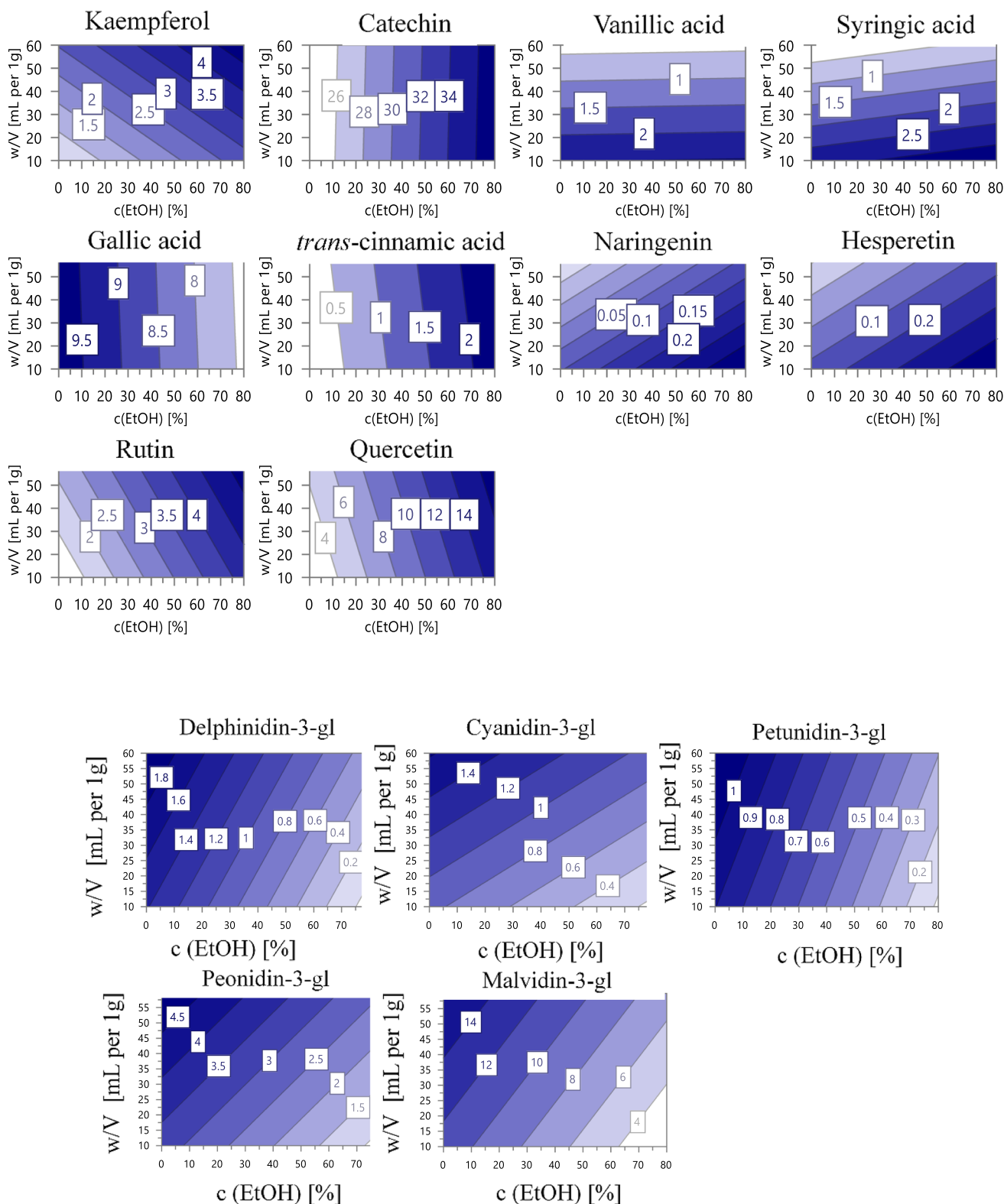


Fig. 2 Contour diagram of the influence of the change of the solvent concentration (x_1) ranged from 0% (i.e. 100% water) to 80% ethanol in water and weight ratio of sample and solvent (x_2) ranged from 1:10 to 1:60 (using fixed US extraction conditions: 55 °C, 30 min) on the individual content (mg/mL) of: kaempferol (y_1), catechin (y_2), vanillic

acid (y_3), syringic acid (y_4), gallic acid (y_5), *trans*-cinnamic acid (y_6), naringenin (y_7), hesperetin (y_8), rutin (y_9), quercetin (y_{10}), delphinidin-3-gl (y_{11}), cyanidin-3-gl (y_{12}), petunidin-3-gl (y_{13}), peonidin-3-gl (y_{14}), and malvidin-3-gl (y_{15}), during the initial screening for extraction conditions using Plackett Burman design of experiments

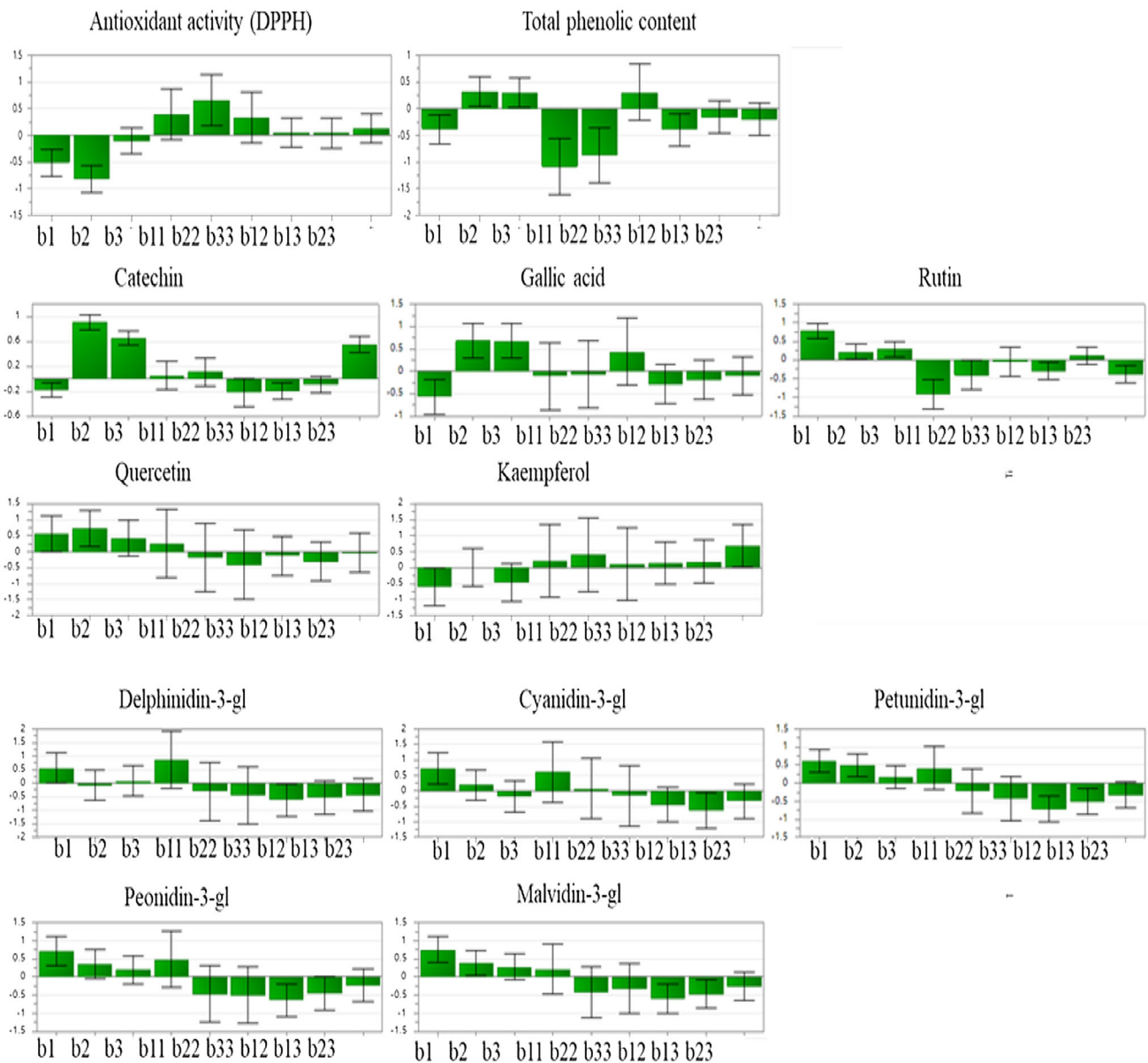


Fig. 3 Normalized coefficients of the 2^3 CCF full factorial DoE showing three effects of different extraction conditions: *b1*—pH value, *b2*—temperature of extraction, *b3*—time of extraction and their interactions (*b11*, *b22*, *b33*, *b12*, *b13*, *b23*) on the extraction

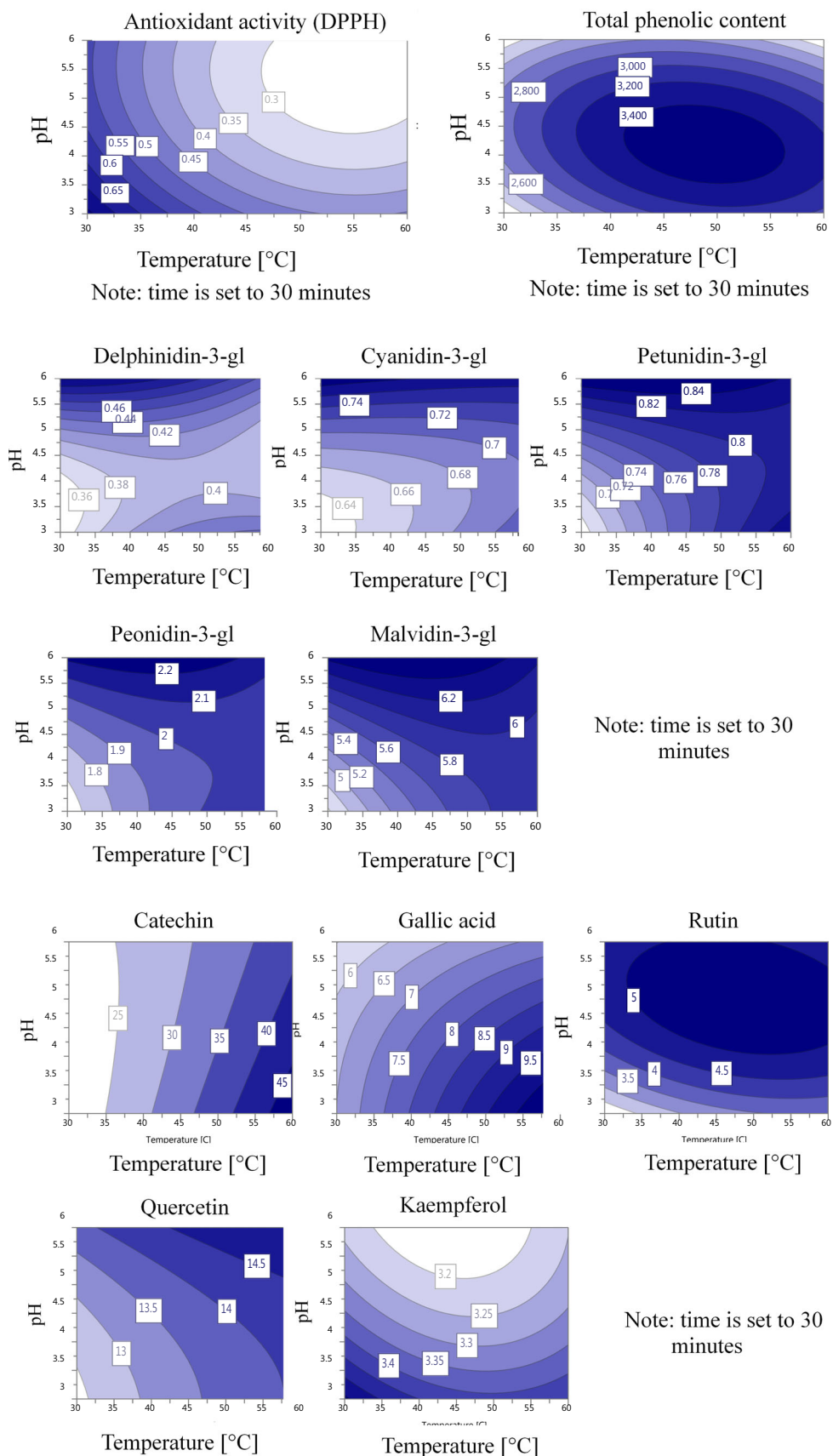
yield descriptors: **A** antioxidant activity, total phenolic content, **B** content of 5 phenols (catechin, gallic acid, rutin, quercetin and kaempferol), **C** content of 5 anthocyanins (delphinidin-3-gl, cyanidin-3-gl, petunidin-3-gl, peonidin-3-gl and malvidin-3-gl)

significant decrease of the phenol concentration and antioxidant capacity of extracts recovered from olive mill wastewater (Galanakis et al. 2010). This reduction could be related to the thermal activation of endogenous polyphenol oxidase. For instance, thermal activation of polyphenol oxidase from other sources like apple occurs between 45 and 65 °C (Soysal 2008). Therefore, the maximum temperature for extracting polyphenols from grape pomace by solid–liquid extraction was 60 °C (Spigno et al. 2007; Pinelo et al. 2005).

As for the anthocyanins content, the best results were obtained at higher extraction temperatures (temperature set above 50 °C showed an excellent yield of all anthocyanins) and pH values above 5 (but also at pH values between 4 and 5).

Better extraction of phenols was achieved at higher temperatures, but the evaluation of the pH value effect on the extraction was not that obvious. For some phenols (rutin and quercetin), better results were achieved at higher pH values and others (gallic acid and kaempferol) at lower

Fig. 4 Contour diagram of the influence of the change of the pH value (x_1) ranged from 3 to 6, temperature of extraction (x_2) ranged from 30 to 60 °C for time set to 30 min on the antioxidant activity (y_1), total phenolic content (y_2), and on the individual content (mg/mL) of delphinidin-3-gl (y_3), cyanidin-3-gl (y_4), petunidin-3-gl (y_5), peonidin-3-gl (y_6), malvidin-3-gl (y_7), catechin (y_8), gallic acid (y_9), rutin (y_{10}), quercetin (y_{11}) and kaempferol (y_{12}), during the optimization of extraction conditions using 2^3 CCF full factorial DoE



pH values. In contrast, the pH value did not influence the extraction of catechin. However, at pH 5 (which proved to be optimal for the descriptors mentioned above: antioxidant activity, total phenolic content, and anthocyanins), the extracted phenols' content was satisfactory when maintaining the temperature at high values. The addition of acids to solvents may increase extraction efficiency. Brazinha and colleagues, in their work, added 3 g/L of citric acid to 60% ethanol, which led to increased yields of individual gallic acid, catechin and epicatechin, and total phenolic content (Brazinha et al. 2014). By using citrate–phosphate buffers (pH 3, 5, and 7) to extract phenols from grape marc, the better antioxidant activity of obtained extracts was achieved at pH 5 and lower (Amendola et al. 2010). It has also been shown that the addition of hydrochloric acid to solvent results in higher total phenolic and anthocyanin content in final extracts (Vatai et al. 2009). Hosseini and colleagues in their work investigated the influence of the addition of three different organic acids to solvent system water/ethanol/organic acid (50:48:2) on the extraction of phenols and anthocyanins from red cabbage, barberry, and eggplant peel. Since the pH of acidified solvent influences the final content of phenols and anthocyanins, the extraction is conducted at constant initial pH (pH = 3.49). Compared with acetic and hydrochloric acid, the addition of citric acid to the solvent system led to extracts' highest total phenolic, anthocyanins content, and antioxidant activity from all three different sources. That could mean that the role of citric acid in the extraction process is dual. Besides lowering the pH of solvents, citric acid may also act as an extractant (Hosseini et al. 2016).

Conclusion

Keeping in mind the overall criteria for extraction efficacy, it was concluded that 55% ethanol, sample/solvent ratio 1:40, pH 4.5, T 55 °C, and time 30 min were the optimal experimental conditions for our development method optimization. Depending on the primary recovery purpose (e.g., targeting antioxidant activity, total phenolic content, individual phenols, or individual anthocyanins), the investigated parameters can be modified accordingly to obtain the desired outcomes. The obtained results are in good correlation with the results available in the literature. Likewise, they covered the optimization of factors not mentioned in the publications (sample/solvent ratio and pH value of extraction solvent). By introducing pure water as an extraction medium, better results were achieved in terms of anthocyanins content. The comprehensive approach for optimizing the extraction procedure enclosed in this research allows for a good insight into the experimental

condition. It leaves an analyst a choice of parameters most relevant for the extraction of interest.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13197-021-05317-9>.

Acknowledgements This work was partially supported by the Ministry of Education, Science and Technological Development of Republic of Serbia (Project No. TR31020). The participation of the authors Turki M.S. Aldawoud and Charis M. Galanakis in this work was supported by Researchers Supporting Project number (RSP-2020/197) King Saud University, Riyadh, Saudi Arabia.

Authors' contributions All listed authors have contributed significantly and equally to the publication. The corresponding authors are available to review 3 or more articles submitted to JFST.

Funding This work was partially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project No. TR31020). The participation of the authors Turki M.S. Aldawoud and Charis M. Galanakis in this work was supported by the Researchers Supporting Project number (RSP-2020/197) of King Saud University, Riyadh, Saudi Arabia.

Declarations

Conflicts of interest Authors would like to declare that they have no conflict or competing interests.

Ethics approval The manuscript is an original work from all authors and has not been published, submitted, or being submitted to another journal.

Consent to participate All authors have read and approved the MS, and all agree of its submission to JFST.

Consent for publication All authors have read and approved the MS, and all agree of its publication in JFST.

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