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Polyphenol extraction from grape wastes: Solvent and pH effect

Celia M. Librán, Luis Mayor, Esperanza M. Garcia-Castello*, Daniel Vidal-Brotons

Instituto Universitario de Ingeniería de Alimentos para el Desarrollo, Universitat Politécnica de València, Camino de Vera s/n, 46022 Valencia, España; *Corresponding Author: egarcia1@iqn.upv.es

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ABSTRACT

World wine industry transforms 10% - 25% of raw grapes into residues, mainly represented by lees, grape marcs, seeds and stems. These by-products are a rich source of polyphenols and therefore they can be used to produce new added value products. The aim of this work was to determine the best process conditions (treatment time, % of ethanol and pH of the solvent) during solid-liquid extraction of polyphenols from grape marcs, by analyzing the phenolic content of the extracts, namely: total polyphenol content, flavanols, flavonols, phenolic acids and anthocyanins. Antioxidant activity of the extracts was also determined. An extraction time of two hours was enough since longer times did not increase process yields. Best extraction yields were obtained for 75% ethanol solutions. Basic pH led to better yields in extracting media with low percentage of ethanol, whereas acid pH presented better extraction yields in extracting media with high percentage of ethanol. Among all the polyphenols extracted, anthocyanins were the most abundant representing over 40% of the total. In general, the best process conditions were 2 h of extraction in a 75% EtOH liquid mixture at pH = 2.

Keywords: Antioxidants; By-Products; Fruits; Solvent Extraction; Wine

1. INTRODUCTION

In 2011, the world wine industry used 13,930,985 tons of grapes for its transformation [1]. Among them, from 10% to 25% (w/w) changed into residues after grape wine processing, being mainly represented by lees, grape marcs, seeds, stems and stalks [2,3]. These wastes are of difficult management due to their high biological oxygen demand [4].

In recent years, scientists have realized of this environmental problem and looked for solutions. Several studies marked these by-products as a rich source of polyphenols and therefore they could be used to produce new added-value products [4-6]. Traditionally, these wastes were used for animal feed but recently they have been found as a low-cost source of antioxidants [7]. Some authors [8] have summarized the health aspects derived from the consumption of phenols from grape, mainly due to their antioxidant activity. Others suggested its application to food to extend their self-life and hence avoiding the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) which use is regulated by international agencies [9,10].

Most common groups of polyphenols found in grapes are: anthocyanins, flavonols, flavanols and phenolic acids [11]. Their total content in grape and grape wastes seemed to not vary among white and red varieties [12] although the extraction procedure has a significant effect on the quantity and quality of extracts [6,7,10]. Since the antioxidant power of grape extracts is in direct relation with their total polyphenol content [5,12], the selection of the best extraction conditions is of great importance, because it could alter the characteristics of the final extract and then have an economic impact.

For these reasons, the aim of this study was to determine the best process conditions (treatment time, percentage of ethanol and pH of the solvent) during solid-liquid extraction of polyphenols from grape marcs, by analyzing the effect of these conditions on several extraction yields, namely on total phenolics, flavonoids, flavanoids, phenolic acids and anthocyanins and also on the antioxidant power of the extracts.

2. MATERIALS AND METHODS

2.1. Grape Marcs

Pressed marcs (from the vinification of Tempranillo red grapes) were provided by the Enology Laboratory of the Institute of Food Engineering for Development-Polytechnic University of Valencia (Spain) and were stored

at −20˚C until their use. Homogeneous samples were taken and thawed at room temperature previous to use them in the experiments. They were dried at 25˚C in a conditioning chamber (ACR-45/87, Dycometal, Spain) up to moisture content of 16% - 18% (wet basis) (determined by dry weight in a vacuum oven (J.P Selecta, VacioTem, Spain) at 70˚C till constant weight) and milled to reach a final particle size between 0.5 and 2.5 mm [7, 10].

2.2. Reagents

Gallic acid, ethanol, methanol, hydrogen chloride and sodium bisulfite were from Panreac. Sodium carbonate was from Fluka. Caffeic acid, *p*-dimethylaminocinnamaldehyde (DMACA) and quercetin were from Sigma. Catechin, Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were from Aldrich. Potassium hydroxide was from AnalaR.

2.3. Extraction Procedure

Solid-liquid extractions were carried out on an orbital shaker (GFL Typ 3005 D-30938 Burgwedel, Germany) at 150 rpm and room temperature (20˚C - 23˚C), with 1/25 (w/v) ratio sample/solvent according to previous studies [13,14]. After the extraction, liquid extracts were separated from solids by centrifugation (3600 rpm for 10 min, Selecta, Medifriger, BL-S, centrifuge), and then stored at −20˚C overnight up to their analysis.

2.4. Extraction Kinetics

Ethanol/water mixtures at different ratios were used as solvents with the necessary amounts of HCl or KOH to regulate the liquid pH (always less than 1 mL). It was necessary to correct the pH lecture by **Eq.1**, because the equipment was calibrated with aqueous tampons.

$$pH = pH_0 + \delta \quad (1)$$

where pH is the corrected lecture and pH_0 is the lecture given by the pHmeter.

The values of δ were obtained from the literature, ranging from −2.9 to 0 [15,16]. The liquid extracts were analyzed for their total polyphenolic index (TPI) at different times during 8 h, for the determination of extraction kinetics.

2.5. Extractions at Fixed Time

A full factorial design [17] with five levels for ethanol concentration (0%, 25%, 50%, 75% and 100%) and four levels for pH (2, 5.3, 8.7 and 12) was used. Experiments were done in duplicate, giving a total of 40 runs. Extraction time was fixed from the previous experiments described in 2.4. The yields for each extracting condition were determined by analyzing the concentration in the extract of total polyphenols, flavonols, flavanols, phenolic acids, anthocyanins and antioxidant activity. All the determinations were done by triplicate.

2.6. Chemical Analyses

2.6.1. Total Polyphenol Index and Total Polyphenol Content

Total polyphenol index (TPI) was determined from the **Eq.2**

$$TPI = A_{280} * n \quad (2)$$

where A_{280} is the absorbance at 280 nm of the extract and n is its dilution factor.

Total polyphenol content (TPC) was calculated from the TPI, standardized against a gallic acid curve expressed as mg gallic acid equivalent (GAE) per mL of extract [4]. Total polyphenol extraction yield was expressed as mg GAE/g dry sample (3).

$$\begin{aligned}&\text{GAE (mg)}/\text{dry sample (g)}\\ &=\left[\text{GA (mg/mL)}\right]*\text{Liquid (mL)}/\text{dry mass (g)}\end{aligned} \quad (3)$$

2.6.2. Total Flavanols

Flavanols were determined after derivatization with *p*-dimethylamino-cinnamaldehyde (DMACA), since this method has proved to have no interferences with anthocyanins. The followed method was adapted from references [12,18]. Briefly, the extract was 1/10 (v/v) diluted with MeOH, and then 1.5 mL of acidified DMACA solution were added to 0.3 mL of methanolic extract. The mixture was allowed to react for 10 min at room temperature, and the absorbance was read at 640 nm. Total flavanol content was standardized against a catechin curve expressed as mg of catechin equivalent (CE) per mL of extract, and the flavanols extraction yield was expressed as mg CE/g dry sample, using an equation similar to **Eq.3**.

2.6.3. Total Flavonols and Phenolic Acids

Total flavonols and phenolic acids were determined following the procedure described by references [7,19, 20]. Briefly, extracts were thoroughly mixed sequentially with acidified ethanol and HCl 2%. Absorbances at 360 and 320 nm were measured for total flavonols and phenolic acids, respectively. After the correspondent calibration curves, the results were expressed as mg of quercetin equivalent (QE) and mg of caffeic acid equivalent (CAE) per mL of extract for total flavanols and phenolic acids, respectively. Both extraction yields were calculated using an equation similar to **Eq.3**.

2.6.4. Total Anthocyanins

Anthocyanins were measured through a chemical me-

thod based on their specific properties of bleaching by SO_2, and calculated by comparison with a standardized anthocyanin solution according to reference [21]. The anthocyanins extraction yield was expressed as mg anthocyanins/g dry sample, using equation similar to **Eq.3**.

2.6.5. Antioxidant Activity

Antioxidant activity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method described by reference [18]. Each extract was diluted 1/10 (v/v) with methanol, and 3.8 mL of DPPH solution (60 µM in MeOH) was added to 0.2 mL of methanolic sample. At t = 0 min ($A_{515}(0)$) and after 30 min ($A_{515}(30)$) of reaction, absorbances were measured at 515 nm and the results were expressed as percentage with **Eq.4**:

$$\%\Delta A_{515} = (A_{515(0)} - A_{515(30)})/A_{515(0)} * 100 \qquad (4)$$

Afterwards, antioxidant activity was expressed as µM of Trolox equivalent per mL of sample, by using a previous calibration curve. The yield was expressed as µmol of Trolox equivalent/g dry sample, and was obtained using and equation similar to **Eq.3**.

2.7. Statistical Analysis

Each extraction, at the different conditions previously explained, was assayed twice, and the obtained extracts were chemically analyzed three times each. Therefore, a descriptive analysis was performed, and all values were averaged and given along with their confidence interval (*t* student). Significant effect of ethanol concentrations and pH were evaluated with an analyses of variance (ANOVA, $p < 0.05$) and a Tukey test was carried out to find differences among groups. Moreover, results were fitted to a second order polynomic equation (**Eq.5**) that considers lineal and quadratic effects as well as interaction effects among the experimental factors studied

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad (5)$$

where Y is the studied response and β_0, β_i, β_{ii} y β_{ij} are the independent, lineal, quadratic and interaction coefficients, respectively. Non-linear fit and goodness of fit (R^2) were performed through the STATGRAPHICS Centurion XVI software (Statpoint Technologies Inc.).

3. RESULTS AND DISCUSSION

3.1. Extraction Kinetics

Figure 1 shows the evolution of TPI with time at different concentrations of ethanol in the extracting liquid (0%, 50% and 100% of ethanol) and without fixing pH (**Figure 1(a)**), with pH = 2 (**Figure 1(b)**) and pH = 12 (**Figure 1(c)**).

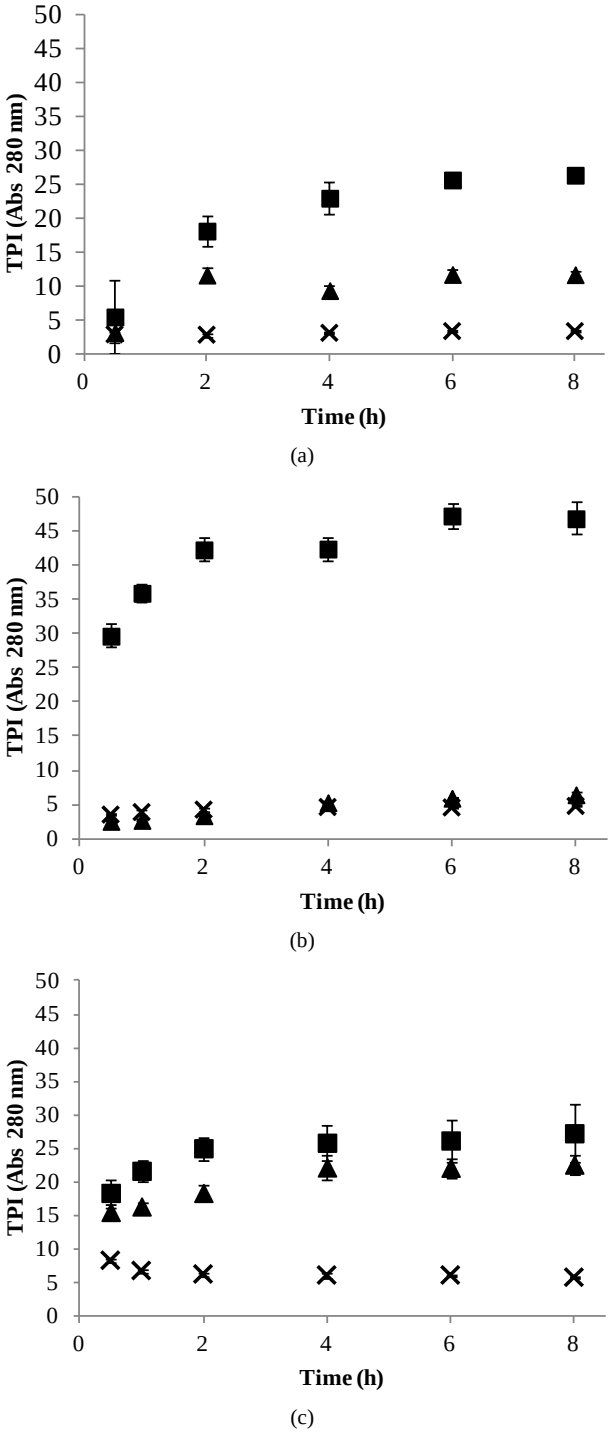

Figure 1. Polyphenol extraction kinetics from wine wastes samples extracted with different water/ethanol mixtures without fixing pH (a), with pH = 2 (b) and with pH = 12 (c) expressed as TPI (mean ± confidence interval). **x**, ■ and ▲: 0%, 50% and100% of ethanol, respectively.

In general, at first stage, the TPI increased fast, followed by a slow increment and then remained practically constant till the end of the process. This asymptotic behavior was found previously by other authors [10,22]. In

Figure 1(b), can be observed that the TPI with 50% EtOH was the highest (twofold the 100% EtOH and sixfold the 0% EtOH) which means a positive effect on the use of this organic solvent until certain concentration. When the pH was fixed, the trend with percentage of ethanol was the same but different behaviour was observed at the different pH assayed. Hence, the acid pH (**Figure 1(b)**) increased the TPI for 0% and 50% EtOH, but decreased it for 100% EtOH and the pH = 12 improved the extraction for 0 and 100% EtOH and did not affect the 50% EtOH.

Different authors marked as better extraction conditions, concentrations of ethanol near to 50% finding decreases on TPI extraction yields with higher EtOH concentrations [6,10,23]. They suggested that ethanol reduces the dielectric constant of the solvent, thus increasing the diffusion of the bioactive molecules with the solvent. However, highly pure organic solvents, e.g. 100% EtOH, could dehydrate the vegetable cells, making difficult the diffusion of polyphenols from the plant material to the extracting liquid.

The pH effect has not been extensively studied before this work. Reference [6] assayed its effect on the stability of extracts. They found that pH 3 and 5 maintain the antioxidant power instead of pH 7 and 9 which showed reductions of this property of the extracts.

All this results showed that indistinctly the pH or the EtOH concentration in the extracting medium, at 2 hours of extraction the TPI yield was at least 90% of the maximum attained during the kinetics experiments. Therefore, this time was used for the next extractions.

3.2. Total Polyphenol Yields

Extraction of total polyphenols (**Figure 2**), according to analysis of variance, was significantly affected ($p < 0.05$) by the ethanol concentration and the pH. Results ranged from 4.58 to 28.06 mg GAE/g dry sample, depending on the extraction conditions (0% EtOH, pH = 2 and 0% EtOH and pH = 12, respectively) and similar results were achieved by reference [10] with 50% EtOH extracting solutions.

It is also observed an increase in the polyphenol extraction yield with basic pH for aqueous extractions (0% and 25% EtOH) and this tendency changed at higher EtOH concentration, where acid pH had the better extraction yields. However, the highest TPCs were obtained with 75% EtOH at all the assayed pH, with exception of pH = 12.

3.3. Flavonol, Flavanol, Phenolic Acid and Anthocyanin Extraction Yields

Figure 3 summarizes the results for the different phenolic compounds identified in the liquid extracts. As observed with the total polyphenols, the results were significantly affected ($p > 0.05$) by both ethanol concentration and pH of the extracting medium.

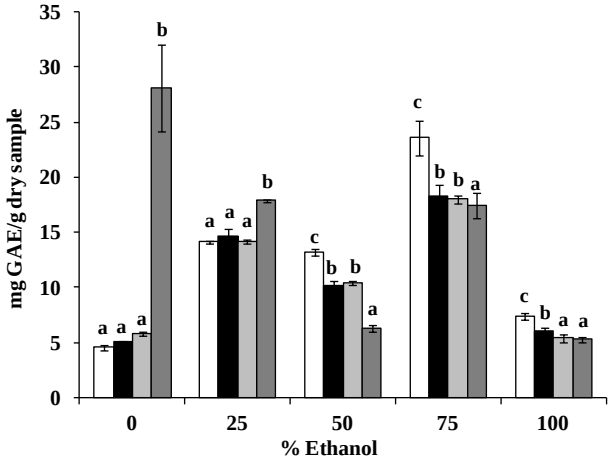

Figure 2. Total polyphenol content yield (mg of GAE/g dry sample, mean ± CI) at 2 hours and 25˚C with different pH and ethanol concentrations in the extracting media. □, ■, ▨ and ▩: pH = 2, pH = 5.33, pH = 8.66 and pH = 12, respectively; a, b, c and d, represent significant differences among groups ($p < 0.05$)

The extraction yields of flavonols, flavanols, phenolic acids and anthocyanins ranged from 0.03 - 4.98 mg QE, 0.09 - 1.83 mg CE, 0.39 - 5.02 mg CAE and 0.85 - 9.83 mg anthocyanins per g of dry sample, respectively. Among the total polyphenols extracted, more than 40% where from the anthocyanin group. Reference [4] studies on polyphenol extraction with water/ethanol mixtures got similar range of values for flavonols and phenolic acids, although slightly lower, and very lower for anthocyanins (almost tenfold less).

In general, all the compounds showed higher extraction yields with higher concentrations of ethanol until 75%. This behaviour could be attributed to the change on polyphenol solubility, density or dielectric constant of the extracting liquid due to the presence of ethanol [20].

Phenolic acids and flavonols extraction (**Figures 3(a)** and **(c)**, respectively) showed similar values. Aqueous solutions (0% and 25% EtOH) get better yields when increasing pH, but higher concentration of ethanol, changed this trend and better yields were achieved with acid pH. Also, flavanols and anthocyanin extractions (**Figures 3(b)** and **(d)**, respectively) showed similar behaviour although this change was found at 25% EtOH.

3.4. Antioxidant Activity

Figure 4 illustrates the antioxidant activity of the liquid extracts. The analysis of variance found a significant effect ($p < 0.05$) of pH and ethanol concentration for all the extraction conditions.

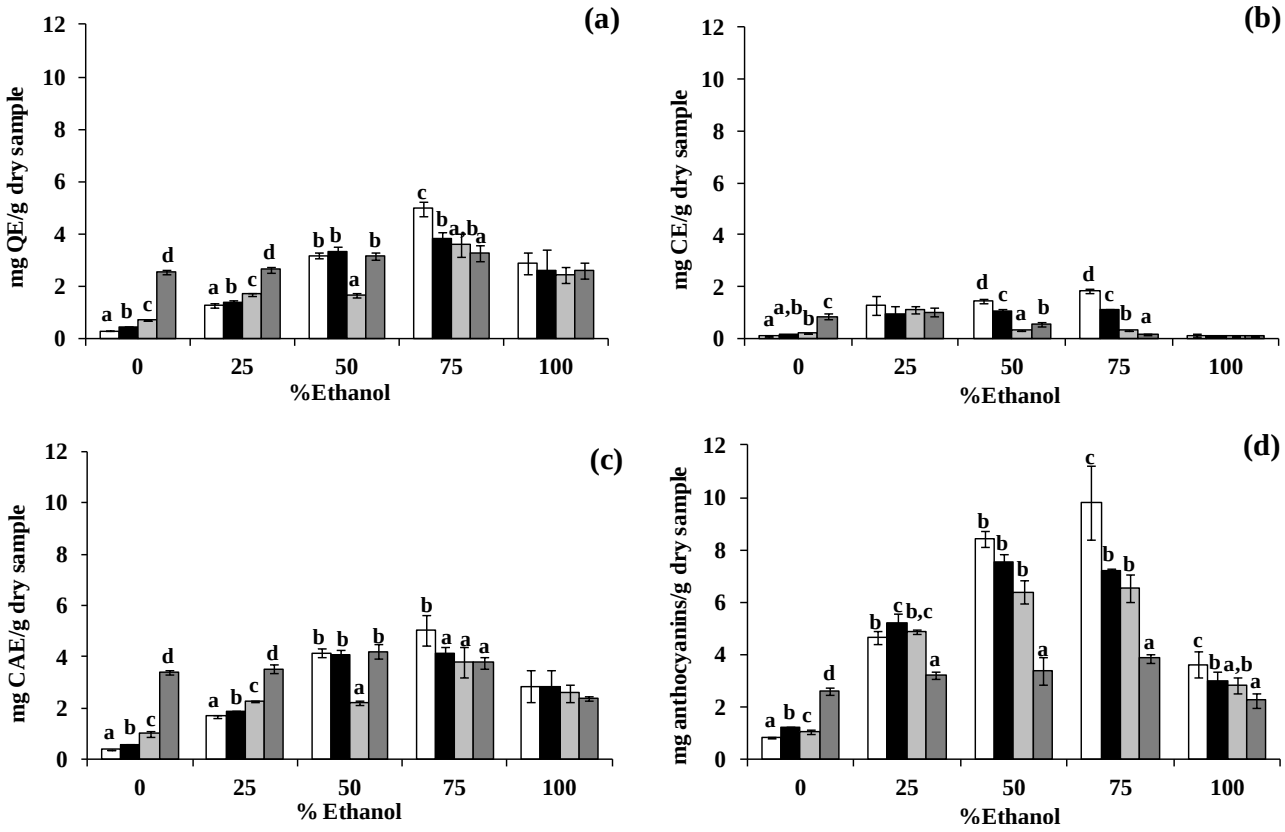

Figure 3. Extraction yields at 2 hours and 25˚C at different pH and ethanol % in the extracting media: (a) flavonols; (b) flavanols; (c) phenolic acids and (d) anthocyanins (mean ± confidence interval). White, light grey, dark grey and black: pH = 2, pH = 5.33, pH = 8.66 and pH = 12, respectively; a, b c and d represent significant differences among groups ($p < 0.05$).

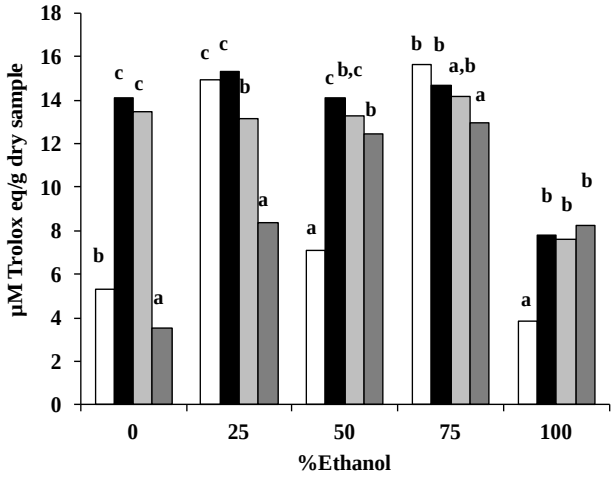

Figure 4. Antioxidant activity at 2 hours and 25˚C with different pH and ethanol % in the extracting media. □, ■, ▒ and ▓: pH = 2, pH = 5.33, pH = 8.66 and pH = 12, respectively; a, b, c and d, represent significant differences among groups ($p < 0.05$).

The extracts from 75% EtOH had the highest antioxidant activity (12.95 - 15.63 µM Trolox/g dry sample) according to the highest polyphenol extraction (**Figure 2**). However little concordance was found in other extracts: 0% EtOH and pH 5.33 and 8.66 showed good antioxidant conditions (14.10 and 13.45 µM Trolox/g dry sample, respectively), although their concentration of polyphenols were not the highest. Previous works indicated the degree of correlation between antioxidant activity and polyphenol contents depends not only on the total polyphenol content, but also on the composition of extracts [4].

Reference [6] recommended pH lower than 5 to preserve the antioxidant activity during storage with 60% EtOH. In general, this fact was in accordance with our results, with exception of 100% EtOH which increased their antioxidant activity at higher pH.

3.5. Response Surface Analysis

Table 1 summarizes the coefficients of the response surface equations and the goodness of fit with the parameter R^2. The values of R^2 were not too high but were similar than those obtained by previous authors on the extraction of polyphenols from different vegetables [20, 24].

Among these results, the most adequate were for anthocyanins (82.39%), phenolic acids (79.59%) and flavonols (76.77%).

Table 1. Coefficients of the response surface equations.

Response (mg/gr·dry sample)	Coefficients*						R^2 (%)
	β_0	β_1	β_2	β_{11}	β_{22}	β_{12}	
Total polyphenols	5.9180	−0.7745	0.4323	0.1268	−0.0033	−0.0162	55.26
Flavonols	0.3447	−0.1502	0.0904	0.0232	−0.0005	−0.0031	76.77
Flavanols	0.6202	−0.1168	0.0376	0.0094	−0.0003	−0.0012	64.98
Phenolic acids	0.3768	−0.1381	0.1117	0.0270	−0.0007	−0.0038	79.59
Anthocyanins	0.8954	0.1604	0.2262	−0.0139	−0.0018	−0.0039	82.39
Antioxidant activity	5.8228	1.8654	0.1608	−0.1586	−0.0022	0.0061	58.20

*Subindexes: 0 = independent term; 1 = pH, lineal term; 2 = % ethanol, lineal term; 11 = pH, quadratic term; 22 = ethanol, quadratic term; 12 = pH*temperature, interaction term.

Response surface plots (not shown) exhibited the trends previously commented in this work. In general, extraction yields increased with higher concentrations of ethanol until 75% and, basic pH improved the extraction of aqueous samples (0% and 25% EtOH) while acid pH was better for ethanol concentrated samples.

4. CONCLUSIONS

This study reflects the importance of controlling the studied extraction conditions (time, pH, % ethanol) to obtain an extract with the highest polyphenol content and with an adequate antioxidant activity.

An extraction time of two hours was enough since longer time did not increase process yields. Best extraction yields were obtained for 75% ethanol solutions. Basic pH led to better yields in extracting media with low ethanol percentage, whereas acid pH presented better extraction yields in extracting media with high ethanol percentage. Among all the polyphenols extracted, anthocyanins were the most abundant representing over 40% of the total. In general, the best process conditions were 2 h of extraction in a 75% EtOH liquid mixture at pH = 2.

5. ACKNOWLEDGEMENTS

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