MICROWAVE PRETREATMENT TO IMPROVE EXTRACTION EFFICIENCY AND POLYPHENOL EXTRACT RICHNESS FROM GRAPE POMACE. EFFECT ON ANTIOXIDANT BIOACTIVITY

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ABSTRACT

Microwave assisted extraction advantages are widely recognised. However, its implementation at industrial scale is restricted due to microwave limitations. In this work, a microwave pretreatment is proposed as an easy scale-up alternative for grape pomace polyphenol extraction, especially for anthocyanins. The double effect of this pretreatment on extraction yield and on product richness is assessed. Microwaves accelerate the extraction kinetics of most compounds, but their effect on polyphenols is more pronounced than in other substances (like sugars and fibres). These differentiated rates are exploited to improve the polyphenol richness of the final dry product. By selecting the appropriate operating conditions, polyphenol yield was increased by 57% and, simultaneously, dry product richness was enhanced by 32%. Also, anthocyanin extraction boost was remarkable. Its content in the final dry product was 85%

higher than the one obtained without the microwave pretreatment. The cellular bioactivity of these extracts was improved by 83% and 133%.

KEYWORDS

Microwave-pressure pretreatment; polyphenols; anthocyanins; extraction yield; richness; antioxidant bioactivity

1. INTRODUCTION

Nutraceutical's demand is growing every year as a consequence of the society concern for chemical preservatives (Frost and Sullivan, 2011; Serra et al., 2008). Since grape pomace is a propitious source of polyphenols, it can be used as a suitable resource to cover this demand of natural preservatives (Lutterodt et al., 2011; Shrikhande, 2000). Polyphenols are widely known for having beneficial health effects due to their ability to subdue free radical induced diseases (Nowshehri et al., 2015). This property makes them highly valuable for applications in cosmetic and food industry; for example, as supplements, food preservatives and natural colourants (Ignat et al., 2011). In particular, polyphenols provide a skin protection that prevents aging and other skin conditions (Yamakoshi et al., 2004). For instance, Wittenauer, et al. (2015) demonstrated the large effectiveness of catechin and procyanidins (polyphenols found in grapes) in the inhibition of collagen-elastase (cause of skin premature aging). In sum, there is a promising niche market for antioxidant products from natural extracts.

Grape pomace is not only a notable source of polyphenols, but also its use as raw material contributes to solve the ecological problem that this residue means (Fontana et al., 2013; Llobera and Cañellas, 2007). According to FAO (2016) and Rombaut (2014), in 2014 winemaking industry produced 5.8 million tonnes of what until recently was considered a valueless residue. However, it has now been appraised as a valuable material that can be used to improve wine industry sustainability. On the other hand, industrial sustainability comes through an effective process, what can be achieved by process intensification. In the case of the extraction of polyphenols from grape pomace, the main drawback to overcome is the bulky

equipment and the long extraction times. Several novel intensification techniques have been studied at laboratory scale, such as ultrasound, high voltage or pressurized extraction (Duba et al., 2015; Goula et al., 2016; Puértolas and Barba, 2016). Between these novel processes, microwave assisted extraction (MAE) has been found to provide very good results (Casazza et al., 2010). Its main advantages are the shortening of the extraction time and the reduction in the amount of solvent used (Li et al., 2013). In addition, Yedhu Krishnan and Rajan (2016) have shown how microwave radiation disrupts the solid structure of the raw material *Terminalia bellerica*, facilitating the leakage of active compounds, what leads to the yield enhancement achieved with MAE. Regarding grape polyphenol extraction, MAE allows to obtain just in 10 minutes twice the amount of polyphenols that is extracted in 3 hours by a conventional solid-liquid extraction (Brahim et al., 2014). Also, the anthocyanin boost is remarkable: 17.6% more of these active compounds are obtained by microwave extraction (Liazid et al., 2011). However, this new extraction technique presents severe scale-up limitations. MAE main disadvantage is its low penetration depth, which for a frequency of 2.45 GHz and the mixture grape pomace-solvent is about 2 cm (Álvarez et al., 2017; Thostenson and Chou, 1999). Therefore, it is not possible to uniformly irradiate a large vessel, as only the external parts will be irradiated. The alternative to increase penetration depth is to use a frequency of 9.15 MHz, which will imply the counterproductive reduction of the thermal effect. That is why, in this work, a short microwave pretreatment is proposed as a preliminary stage to the conventional extraction, instead of a full time MAE process. A low residence time pretreatment will allow the material to be homogeneously irradiated in a size-reasonable microwave oven. In addition, by using an intense but short microwave pretreatment, the material will be able to absorb an amount of energy equivalent to MAE milder radiation conditions. In fact, Yuan Li et al. (2012) pointed out that the total energy absorbed by the material was more relevant than the power irradiated. Thus, both intensification processes can be considered equivalent in terms of matrix structure disruption. The peak of energy during the pretreatment causes a sharp temperature increase. But, because of its short duration, the degradation of active compounds is avoided (Sólyom et al., 2014), while the microwave thermal effect that accelerates the extraction is maintained. Once

the disruption of the cell wall is accomplished, extraction continues as a conventional solid-liquid extraction to assure the leakage of the remaining active compounds.

Another important aspect to consider is the quality of the extract. The improvement on the extraction has to be in quantity (process yield) and in quality (product richness), understanding as product richness the concentration of active compounds in the final product. This value should be reported on a dry basis, since this is the state in which the final product is commercialised in order to assure its stability (de Souza et al., 2015). Final product polyphenol richness is of crucial relevance for its commercialization since the higher the polyphenol richness, the more valuable the product is. Undesired substances, like sugars and fibres that are also obtained in the extraction, depreciate the final product, and also interfere in its biofunctionality. These interactions may sometimes be positive, whereas in other cases they hinder polyphenol antioxidant capacity. For instance, Benayad et al. (2014) reported that polyphenol concentration is not proportional to antioxidant activity due to structure difficulties during oxidation patterns because of macromolecule steric hindrances. On the other hand, Serra et al. (2008) found that grape extracts present a larger antimicrobial activity than single polyphenol solutions with the same equivalent concentration. A synergetic effect between polyphenol and other substances was reported.

In sum, polyphenol's bioactivity not only depends on its concentration, but also on its accessibility (Benlloch-Tinoco et al., 2015). Polyphenols are associated with cell wall molecules, such as lipids, proteins, and sugars (Pérez-Jiménez et al., 2014). So, these compounds are the most likely to interfere in polyphenol activity. Lipid interaction is important regarding digestion, as it restrains lipid absorption (Jakobek, 2015). Several articles have been published about this. An example is the prevention of weight increase in mice with a fat dietary supplemented with black tea polyphenols (Uchiyama et al., 2011). In the case of proteins, the interaction conceals the polyphenol properties. Hasni et al. (2011) demonstrated that the binding between tea polyphenols and milk proteins makes both molecules unable to perform their functions. Sugars, instead, are able to encapsulate polyphenols and deliver them where their

ability to quench free radicals is more useful (Jakobek, 2015). For instance, sugars can protect polyphenols through the gastrointestinal parts and release them in the colon, creating a positive antioxidant growth medium for microflora (Jakobek, 2015; Saura-Calixto, 2011). However, hampering effects have also been described, such as the bioactivity reduction caused by ferulic acid and arabinoxylands bonding (Adam et al., 2002).

The objective of this work is to develop an intensification step easy to be implemented at industrial scale to enhance the conventional solid-liquid extraction process. Extraction conditions are studied in order to fulfil industrial requirements, like solvent consumption reduction and the production of a polyphenol rich extract. Process yield and product richness are analysed together to optimise the extraction conditions. In addition, both chemical and cellular antioxidant activity assays are performed to assess whether the microwave pretreatment has any effect on final product antioxidant capacity.

2. MATERIALS AND METHODS

2.1. MATERIALS

Grape pomace from red *Tempranillo* grape was kindly given by Bodega Matarromera (Spain). It corresponded to 2014 vintage. Grape pomace was stored at -18ºC to preserve its antioxidant activity. Samples were thawed overnight at 4ºC before use. A mixed solvent of 50% ethanol and acid water was employed for extraction. Water was acidified with sulphuric acid to pH=1 in order to improve polyphenol stability.

Chemical reagents Folin-Ciocalteu, potassium chloride, sodium acetate, and sodium carbonate were purchased from Panreac Química (Barcelona, Spain). HPLC standards were bought in Extrasynthese (Lyon, France). Cell culture media and supplements, namely RPMI 1640-medium, fetal bovine serum (FBS), penicillin-streptomycin and trypsin/ethylenediamine tetraacetic acid (EDTA) were purchased from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK). For cytotoxicity and antioxidant activity assays, phosphate buffer solution (PBS) was prepared using the phosphate buffered saline powder, 3-(4,5-dimetylthiazol-2-yl)-5-(3-

carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2´,7´-dichlorofluorescin diacetate (≥ 97%), quercetin (≥ 97%) and 2,2′-azobis (2-methyl-propionamide) dihydrochloride (AAPH, 97%) were provided by Sigma-Aldrich (St Louis, MO, USA).

2.2. EXTRACTION PROCEDURE

An industrial process currently running was used as a control and secondary process to assess the effectiveness of a microwave pretreatment (Moro González, 2009). Laboratory conditions to reproduce industrial conventional solid-liquid extraction were as follow. The amounts of 30 grams of grape pomace and 60 mL of mixed solvent were poured in a 100 mL round bottom flask. A 5 minute homogenization (stirring at ambient temperature) was performed before the extraction. The extraction was set to begin when the flask was introduced in a water bath at 60ºC with vigorous shaken (750 rpm). The solid-liquid extraction was carried out for 3 hours.

For the extractions were microwaves were used, the microwave pretreatment was implemented between the homogenization and the extraction stages. A commercial monomode oven working at a frequency of 2.45 GHz, CEM Discover One microwave (CEM Corp.), was used. A different solid-liquid ratio than in the conventional extraction was employed. For the pretreatment, 30 grams of grape pomace were mixed with 40 mL of solvent. A preliminary analysis of the solid-liquid ratio during the pretreatment suggested the convenience of using a higher ratio during the microwave pretreatment than in the subsequent conventional extraction (0.75 and 0.50 g/mL, respectively). It was found that a ratio of 0.75 g/mL during the microwave pretreatment fulfils a balance between mass transfer and energy efficiency. This is owing to the fact that this ratio provides enough solvent for the diffusion forces to continue to impel the leakage of polyphenols, but the media is not so diluted that the microwave effect is attenuated. After being homogenised, the mixture was irradiated with 300 W of power for 30, 60, 120 and 150 seconds, so pretreatment temperatures of approximately 60, 80, 100 and 120ºC were achieved. These energy densities have been labelled as MP60, MP80, MP100 and MP120, respectively. Temperature was continuously recorded with an optic fibre thermometer

(FoTEMP 4, OPTPcon GmbH), calibrated in an ice water bath. For the MP100 and MP120 pretreatments, a glass pressure vessel (QianCap, QLabtech) was used to maintain the solvent in a liquid phase under pressure. Figure 1 represents the energy absorbed during each pretreatment. It includes a Control experiment that represents the conventional solid-liquid extraction, in which no pretreatment took place. Energy absorbed was computed by the sum of the sensible and latent heat. Heat losses to the environment were dismissed, since Sólyom et al. (2011) proved its low contribution. The efficiency between the emitted and the absorbed energy was found to be 58%.

When irradiation was over, 20 mL of solvent were added to reach the same ratio used in the conventional solid-liquid extraction process. The grape pomace-solvent mixture was quickly cooled down to 60ºC in an ice bath, and it continued with the conventional solid-liquid extraction in a water bath.

Liquid samples were taken during the extraction at different times to perform a kinetic study. The start of the extraction was set after the 5-minute homogenization (t=0). The initial concentration (C_0) represents the released of non-bound polyphenols. After the homogenization, samples were taken after the pretreatment and during the subsequent conventional process. Extraction kinetics followed a first order equation, like shown in expression (1); where C_{cal} stands for the calculated concentration in mg/g at a time t (minutes), C_0 represents initial concentration (mg/g), C_f is a pre-exponential factor (mg/g) that together with C_0 (C_0+C_f) corresponds to extraction yield ($t = \infty$), and k is the rate extraction constant (min^{-1}).

$$C_{cal} = C_0 + C_f \cdot [1 - \exp(-kt)] \quad (1)$$

Concentrations have been expressed in two different ways: as mg/g of dry pomace and as mg/g of dry extract. The first one, $mg/g_{Dry\ Pomace}$, represents the extraction yield: the amount of polyphenols extracted from the raw material. The second, $mg/g_{Dry\ Extract}$, describes the final dry product richness, as it indicates the ratio between polyphenols and the total extracted substances.

Expression (1) correlation parameters C_0, C_f and k have been adjusted by minimizing the average relative deviation (ARD) between the experimental concentration and the calculated value, as equation (2) shows. Regressions have been performed for yield and for richness results. In the case of extract richness, the extraction of other compounds must be also considered. That is why richness regression curve has to be computed as the ratio between the concentration of active compounds and the total amount of substances extracted (dry extract residue).

$$ARD = \frac{1}{n}\sum_{i=1}^{n}\left|\frac{C_{\exp} - C_{cal}}{C_{\exp}}\right| \quad (2)$$

Experiments were performed in triplicate to assure the reproducibility of the process. Results have been expressed as mean ± standard deviation.

2.3. SOLID RESIDUE

The dry extract residue was measured gravimetrically by drying extracts at 85ºC during 48 hours.

2.4. POLYPHENOL CHARACTERIZATION

2.4.1. TOTAL POLYPHENOL CONTENT

Total polyphenol content was measured by Folin-Ciocalteu method (Waterhouse, 2001). Briefly, 40 μL of diluted sample were mixed with 3000 μL of distillate water and 200 μL of Folin-Ciocalteu reagent. After 5-minute shakening, 600 μL of 20% sodium carbonate were added. Samples were left 30 minutes at 40ºC. Then, absorbance was measured at 765 nm (Shimadzu UV/VIS Spectrophotometer). Results were expressed in gallic acid equivalents (mg_{GAE}/g).

2.4.2. ANTHOCYANIN CONTENT

Anthocyanin content was determined by the pH differential method (AOAC, 2005). Samples were diluted in two buffers (potassium chloride 0.025 M pH=1 and sodium acetate 0.4 M pH=4.5). Absorbance was calculated as the increment in absorbance at 520 minus the increment at 700 nm between the two buffers. An extinction coefficient of 26900 L/mol/cm was used. Results were expressed as cyanidin-glucoside equivalents (mg_{CGE}/g).

2.4.3. IDENTIFICATION AND QUANTIFICATION OF POLYPHENOLS (HPLC)

Gallic acid, catechin, procyanidin B2, epicatechin, myrecetin, quercetin and malvidin were quantified on a Waters e2695 Separation Module equipped with a quaternary pump and a Waters 2998 photodiode array detector (DAD). A method for grape and wine phenols was used (Gómez-Alonso et al., 2007). A C18 guard colum OptiGuard 1 mm (Sigma-Aldrich, Spain) and a C18 column Mediterranea Sea 5 μm 250x4.6 mm (Teknokrima, Spain) were employed. Temperature was kept at 30ºC and 1 mL/min of the ternary eluent was pumped. Eluent's gradient is specified in Table 1. Samples were filtered with 0.45 μm regenerated cellulose syringe filters and a sample volume of 20 μL was injected in the system. DAD signals were recorded at 280, 320, 360 and 520 nm. UV/VIS spectra was also registered. Waters Empower 3 software acquired the data and allowed the processing. Compound identification was made by comparing retention time and UV/VIS spectra of each polyphenol with standards.

2.5. OTHER SUBSTANCES DETERMINATION

2.5.1. NON-POLYPHENOL CONTENT

Non-polyphenol content was computed by subtracting the concentration of polyphenols to the total amount of substances extracted, determined by the dry solid residue.

2.5.2. SUGAR CONTENT

Sugars were determined by the anthrone method. In a 70% (v/v) sulfuric acid solvent 2 g/L of anthrone reagent were dissolved. A volume of 5 mL of this solution was mixed with 1 mL of

sample, cooled down in an ice bath, and thorough shaked for 15 s. Then, the samples were put 10 minutes in a boiling water bath. After cooling them down, absorbance at 625 nm was recorded. Results were quantified by comparison with a glucose calibration curve, and so, results were expressed as glucose equivalents (mg_{GE}/g).

2.5.3. FIBRE CONTENT

Dietary fibre was computed spectrophotometrically at 320 nm, using an absorptivity coefficient of 5.7 L/g/cm (Prozil et al., 2014). This value was corroborated by an external laboratory assay (Agrolab, Spain).

2.6. CHEMICAL ANTIOXIDANT ACTIVITY

Chemical antioxidant activity was measured by the oxygen radical absorbance capacity assay (Ou et al., 2001). It measured the sample capacity to quench peroxyl radicals from α,α′-azodiisobutyramidine dihydrochloride (AAPH) by inhibiting fluorescein sodium oxidation. Phosphate buffer (10 mM, pH=7.4) was used as solvent for all the solutions. Trolox standards were employed to quantify the antioxidant capacity. A 96-well plate was filled with 25 μL sample (trolox standard or diluted extract) and 150 μL fluorescein sodium salt (100 nM). The plate was incubated at 37ºC for 30 minutes. Reaction was started by the addition of 25 μL of AAPH 240 mM. Fluorescence was recorded by a BMG LABTECH Fluostar OPTIMA fluorescent reader at the emission wavelength of 530±25 nm and excitation wavelength of 485±20 nm, during 90 minutes. Each sample was measured at least six times. Antioxidant capacity was computed by a calibration obtained from the area under the oxidation curve of trolox standard solutions. Results were expressed as trolox equivalents per gram of dry extract ($\mu mol_{Trolox}/g_{Dry\ Extract}$).

2.7. CELL ASSAYS

2.7.1. CELL CULTURE

Human colon cancer cell line, Caco-2, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Barunshweig, Germany). The cell line was grown in RPMI 1640 medium supplemented with 10% (v/v) of FBS and 5000 units of penicillin-streptomycin. Stock cells were maintained as monolayers in 75 cm^2 culture flasks and subcultured at a split ratio of 1:4 by treatment with 0.1% trypsin/0.02% EDTA and incubated at 37 ºC in a 5% CO_2 humidified atmosphere.

2.7.2. CYTOTOXICITY ASSAY

Toxicity assays were performed as previously described by Serra et al. (2013) using completely confluents Caco-2 cells which are a good model of the intestinal barrier (Sambuy et al., 2005). Briefly, Caco-2 cells were seeded at a density of 2×10^4 cells/well in 96-well plates and the medium was changed every 48 h. After achieving confluence (5-7 days), cells were exposed with different concentrations of the grape pomace extracts, dissolved in culture medium (RPMI 1640-medium with 0.5% (v/v) FBS) and incubated during 4 hours at 37 ºC and 5% CO_2. After the incubation time, the medium with different sample concentrations was removed and cells were washed with PBS twice. Then, 100 µL of MTS dissolution in RPMI 1640-medium with 0.5% FBS (16.6% (v/v) of a stock solution of 1:10 (pure MTS: RPMI 1640-medium with 0.5%FBS) was added to each well and the 96-well plate was incubated during 2 hours. The MTS assay is based on the conversion of a tetrazolium salt into a coloured, aqueous soluble formazan product by mitochondrial activity of viable cells at 37°C. The amount of formazan produced by dehydrogenase enzymes is directly proportional to the number of living cells in culture and can be measured at 492 nm (Malich et al., 1997). The amount of formazan produced was quantified spectrophotometrically at 492 nm in a microplate reader (EPOCH, Bio-Tek, USA). Results were expressed as percentage of cellular viability relative to the untreated control (cells with RPMI 1640-medium with 0.5% (v/v) FBS). In order to achieve reproducibility in the results, experiments were performed in triplicate in three independent assays.

2.7.3. CELLULAR ANTIOXIDANT ACTIVITY

In order to evaluate the cellular antioxidant activity of the grape pomace extracts, Caco-2 cells were seeded at a density of 2×10^4 cells/well in 96-well plates and the medium was changed every 48 h. The experiments were performed using completely confluents cells (5-7 days). Intracellular antioxidant activity of the different grape pomace extracts was evaluated following the formation of reactive oxygen species in Caco-2 cells after treatment with a chemical stress inducer (AAPH). The formation of intracellular reactive oxygen species was monitored using the fluorescent probe, DCFH-DA, as described by Wang and Joseph (1999) and Serra et al. (2010). Briefly, confluent Caco-2 cells were washed with PBS twice. Then, cells were exposed with different concentrations of the different extracts dissolved in PBS (50 μL/well) and with 50 μM DCFH-DA (50 μL/well), to be later incubated during 1 hour at 37 ºC in a 5% CO_2 humidified atmosphere. Quercetin was used as standard. After the incubation time, the medium was removed and the APPH solution with a concentration of 0.6 mM was added (100 μL/well). Fluorescence was measured for each sample between 0 and 60 min in a FLx800 BioTek fluorescence microplate reader. Cellular antioxidant activity of the extracts was quantified according to Wolfe and Liu (2007) and expressed as quercetin equivalents per gram of dry extract ($\mu mol_{Quercetin}/g_{Dry\ Extract}$). In order to achieve reproducibility in the results, experiments were performed in quadruplicate in four independent assays.

3. RESULTS AND DISCUSSION

3.1. YIELD AND RICHNESS IMPROVEMENT

Current running conventional solid-liquid extraction processes are carried out during long extraction times to deplete the raw material and so, obtain a high polyphenol yield. However, it also compromises polyphenol richness, as Figure 2(A) proves. Long times entail the extraction of undesired substances that reduce product richness. This is clearly evident in anthocyanin extraction, as shown in Figure 3(A). The maximum anthocyanin richness was reached at 60 minutes, where only the 79% of total anthocyanins and 86% of total polyphenols had been

extracted. By continuing the extraction to 180 minutes, all the available polyphenols were obtained, but anthocyanin richness decreased a 31% (from 10.4±2.6 $mg_{CGE}/g_{Dry\ Extract}$ at 60 minutes to 7.2±0.7 $mg_{CGE}/g_{Dry\ Extract}$ at 180 minutes). Therefore, conventional extraction did not allow to obtain maximum yield and richness simultaneously at any operation conditions, neither for polyphenols nor for anthocyanins.

The addition of a microwave-pressure pretreatment to the conventional solid-liquid extraction accelerated polyphenol extraction. Higher yields were obtained at shorter times: between 42-66% more polyphenols were extracted per gram of raw material. Taking into account product richness, the extraction of other undesired compounds was also affected by the radiation, but not in such a significant way as polyphenols did. These two characteristics, the polyphenol extraction acceleration and the different extraction rate of other compounds, made possible to find an optimal time where both yield and richness were maximum.

Extraction kinetic parameters for each pretreatment studied are detailed in Table 2. Richness regression curve must be computed as the fraction between the concentration of active compounds and the total extracted substances (total polyphenols and non-polyphenols present in the dry residue). This is due to the necessity of taking into account the extraction kinetics of the active compounds and of the non-polyphenols.

MP120, MP100 and MP80 pretreatments allowed to obtain the maximum yield in shorter extraction times. The lowest energy pretreatment tested, MP60, did not show a large microwave improvement for total polyphenol content. It presented similar kinetics to the control experiment, in which yield increased with extraction time. This difference between MP60 with the rest of the pretreatments can be clearly seen in the values of the extraction rate constant (k). MP60 and Control extraction rate constant are one order of magnitude lower than the constants of the rest of the pretreatments. Regarding product richness, the same distinction between high energy pretreatments (MP120, MP100, MP80), and MP60 and Control experiments was found. In the first group, extract polyphenol richness soared, achieving its maximum after 10-30 minutes of extraction. Then, it slightly decreased when extraction carried on. The combination

of pressure and microwave radiation up to a temperature of 100ºC (MP100 pretreatment) provided the best results in terms of total polyphenol. Figure 2 shows the extraction kinetics of MP100 pretreatment compared to conventional extraction. As it can be observed, all the extractable polyphenols were obtained after the pretreatment, there was no further yield improvement in the ensuing conventional extraction. A 48% higher yield was obtained as well as a 7-fold extraction time reduction. In addition, by stopping the extraction after 40 minutes, it was possible to increase product polyphenol richness without sacrificing yield. At this time, the richness was maximum (261.5±2.5 $mg_{GAE}/g_{Dry\ Extract}$) and it was 1.3 times higher than the conventional extraction process. If the extraction continued, no more polyphenols were extracted. Instead, richness steadily decreased.

Particular attention deserves anthocyanin extraction. The dilution effect due to undesired extracted compounds was more noteworthy in this group. Anthocyanins are mainly located in grape skin, so, its extraction does not have great mass transfer limitations to overcome (Pinelo et al., 2006). This led to a fast anthocyanin extraction during both conventional and pretreatment processes. As in polyphenols extraction, this faster release enables to stop the processes when yield and richness are at their maximums. The most favourable time to obtain rich extracts was placed at the early stages of the process for pretreated extractions (20-30 minutes), and at the middle of the process for the conventional extraction (60 minutes). In the case of the combination of pressure and microwave pretreatment (MP120 and MP100), the decrease in anthocyanin richness was quite significant. Reductions of 66% and 50% were found by no stopping the extraction in the early stages, and letting it continue for longer. These high energetic pretreatments are not recommendable for anthocyanin extraction, even though no fruitless effects, such as degradation, has been observed (yield remained steady at its maximum along the extraction). In contrast, the lowest energy pretreatment, MP60, also provided low anthocyanin richness, although this result was due to the low efficiency that this pretreatment has been proved to have. To obtain an anthocyanin rich product, the MP80 pretreatment is

recommendable. It was possible to get an 85% more concentrated extract than the one obtained by conventional extraction by stopping the process at 30 minutes, as Figure 3(B) demonstrates.

The richness peaks found during the extractions corroborated the importance of stopping the process at a proper time. Figure 4 attests that the dilution effect is due to the leakage of other substances. The addition of the pretreatment accelerated the release of non-active compounds. However, this enhancement had a lower pronounced effect than the acceleration observed in polyphenol extraction. This differentiated effect of microwaves between each group led to find an optimal time for richness, in which polyphenols were completely extracted, but undesired substances were still being released. The inefficiency of the combined pretreatment of microwaves and pressure (MP120 and MP100) for non-polyphenols was conspicuous. While only microwave pretreatments (MP80 and MP60) improved the yield of non-active compounds over conventional extraction, microwave-pressure pretreatments presented a low efficiency for these undesired compounds. Of these non-active compounds, sugars and fibre extraction have also been quantified. The kinetic parameters obtained are gathered in Table 2. A maximum of 127.8±27.8 $mg_{GE}/g_{Dry\ Extract}$ and 256.9±13.3 $mg_{Fibre}/g_{Dry\ Extract}$ were obtained for sugars and fibres, respectively.

3.2. SELECTION OF THE OPTIMAL EXTRACTION CONDITIONS AND REPRESENTIVE EXTRACTS

In the previous section, the importance of knowing the extraction kinetics in order to select the optimal conditions to enhance the process was exposed. In the present section, a representative sample of each extraction process has been selected to assess its functionality. Selection was performed attending to product quantity and quality. Table 3 summarizes the characterization of each sample further analysed.

Chromatography analysis revealed the same pattern found for the extraction of total polyphenols and anthocyanin for each key polyphenol analysed. As Figure 5 shows, MP100 and MP80 are the pretreatments that greatly enhance the output.

3.3. CELL VIABILITY

Cell viability was not affected up to doses of 10 $mg_{GAE}/g_{Dry\ Extract}$ in any of the extracts tested. However, Figure 6 clearly shows the dose dependent toxic effect of the high dose extracts, except for MP80 whose effect on cell viability stood for longer. It may be due to the large concentration of anthocyanins, particularly malvidin, which may play a role as a cytoprotective agent. Especial attention deserved MP120 and MP100, since cell proliferation was improved by small doses of these extracts. This could be explained by their lower content in non-polyphenols, which can be associated to cellular toxicity. A more prolonged exposure of the matrix at higher temperature might increase the extract concentration in toxic degradation products and also affect the cytoprotective action of specific polyphenols such as anthocyanins (Choi et al., 2007).

3.4. CHEMICAL AND CELLULAR ANTIOXIDANT ACTIVITY

Antioxidant activity is the main feature of this kind of extracts. The extracts capacity regarding scaveging of peroxyl radicals were evaluated by two distinct assays. First, a chemical assay was performed and the results compared with the extracts activities at cellular level. Results can be seen in Figure 7.

Chemical antioxidant activity was proportional to the concentration of polyphenols, but cellular antioxidant capacity did not present this direct relation. Regarding cellular antioxidant activity, MP120, MP60 and Control provided similar antioxidant capacities. In contrast, MP100 and MP80, the richest polyphenol and anthocyanin extracts, had the highest antioxidant bioactivity: 133% and 83% more, respectively, than the conventional extract. MP100 had the lowest

proportion of non-polyphenol substances, so, the interference that these compounds may cause in the absorption of the active compounds may be subdued. On the other hand, MP80 enhancement can be attributed to the high antioxidant activity of anthocyanins. Their large capacity may help to overcome limitations that restrict other samples.

Therefore, MP100 and MP80 pretreatments not only improved the extraction process and the product quality, but also its bioactivity. A characteristic that is considered an asset in the development of commercial products.

4. CONCLUSIONS

In terms of process efficiency, the addition of the here proposed microwave pretreatment allowed to improve extraction yield and polyphenol richness, both at the same time. The acceleration effect of microwaves on extraction kinetics was more pronounced on polyphenols than on other compounds, such as sugar and fibres. This result led to an optimal extraction operation time when polyphenols had already been fully extracted, but those other substances that reduce polyphenol richness were still being released. At that time, the maximum yield and richness were achieved simultaneously. Conversely, in conventional extraction maximum yield and maximum richness were achieved at different extraction operation times, which made it impossible to achieve both objectives concurrently.

Two different pretreatments have been selected to optimise polyphenol or anthocyanin extraction. Regarding total polyphenol content, a 120 seconds pretreatment (MP100) was found to provide the highest polyphenol yield and richness, by a combination of microwave and pressure up to a temperature of 100ºC. The extract yield was improved by 48% and the final dry product richness by 35%, using only one seventh of the extraction time required in the conventional process. If the extraction objective were anthocyanins recovery, the optimal microwave pretreatment time would be 60 seconds (MP80), and the extraction media would be heated up to 80ºC. Microwaves provided high efficiency to the anthocyanin extraction process,

what significantly soared final dry product richness, and allowed to obtain an 85% richer anthocyanin extract.

Both pretreatments (MP100 and MP80) not only improved process efficiency, but also product bioactivity. It was found that these polyphenol and anthocyanin rich extracts presented a more pronounced cellular antioxidant activity against peroxyl radical damage. The final product bioactivity is doubled by the addition of the microwave pretreatment.

In sum, the microwave pretreatments here proposed improved product quality and quantity, as well as extract biofunctionality.

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FIGURE CAPTIONS

Figure 1. Specific energy absorbed during the microwave extraction pretreatment.

Figure 2. Polyphenol extraction richness and yield for (A) conventional extraction and (B) MP100 pretreatment. ● Polyphenol richness; ■ Polyphenol yield.

Figure 3. Anthocyanin extraction richness and yield for (A) conventional extraction and (B) MP80 pretreatment. ● Anthocyanin richness; ■ Anthocyanin yield.

Figure 4. Non-polyphenol extraction kinetics. ● MP120, □ MP100, ▲ MP80, ○ MP60, ■ Control.

Figure 5. HPLC analysis of the representative extraction samples according to Table 3.

Figure 6. Cell viability. ● MP120, □ MP100, ▲ MP80, ○ MP60, ■ Control.

Figure 7. Chemical and cellular antioxidant activity against peroxyl radical.

FIGURES

Figure 1

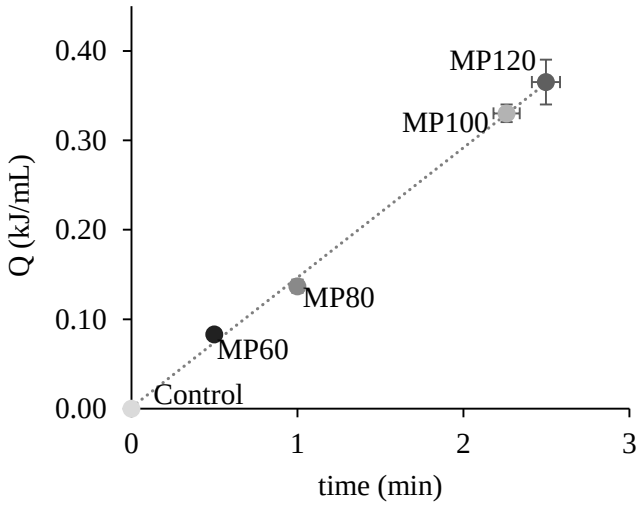


Figure 2

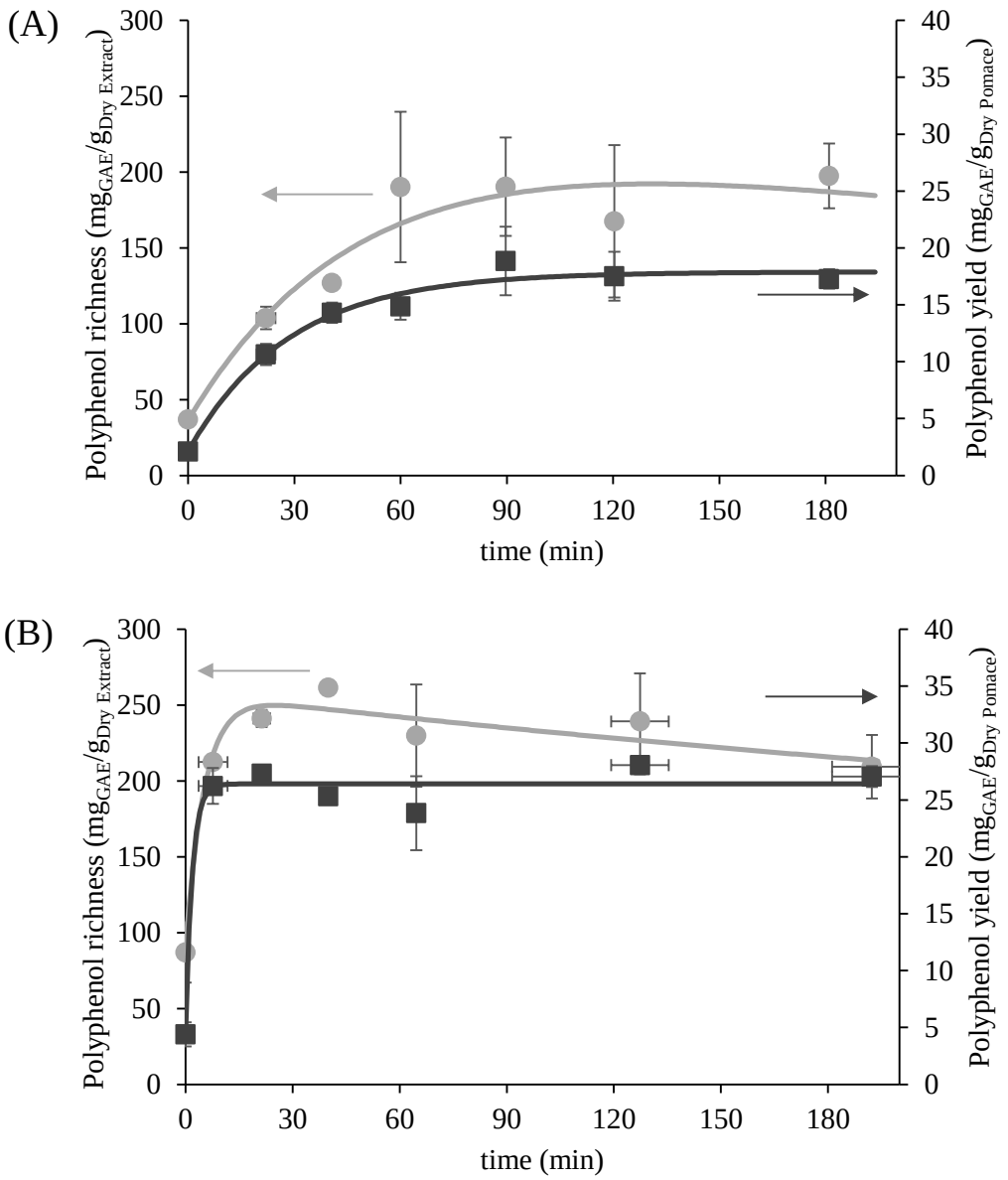

(A)
Polyphenol richness (mg$_{GAE}$/g$_{Dry\ Extract}$)
Polyphenol yield (mg$_{GAE}$/g$_{Dry\ Pomace}$)
time (min)
(B)
Polyphenol richness (mg$_{GAE}$/g$_{Dry\ Extract}$)
Polyphenol yield (mg$_{GAE}$/g$_{Dry\ Pomace}$)
time (min)

Figure 3

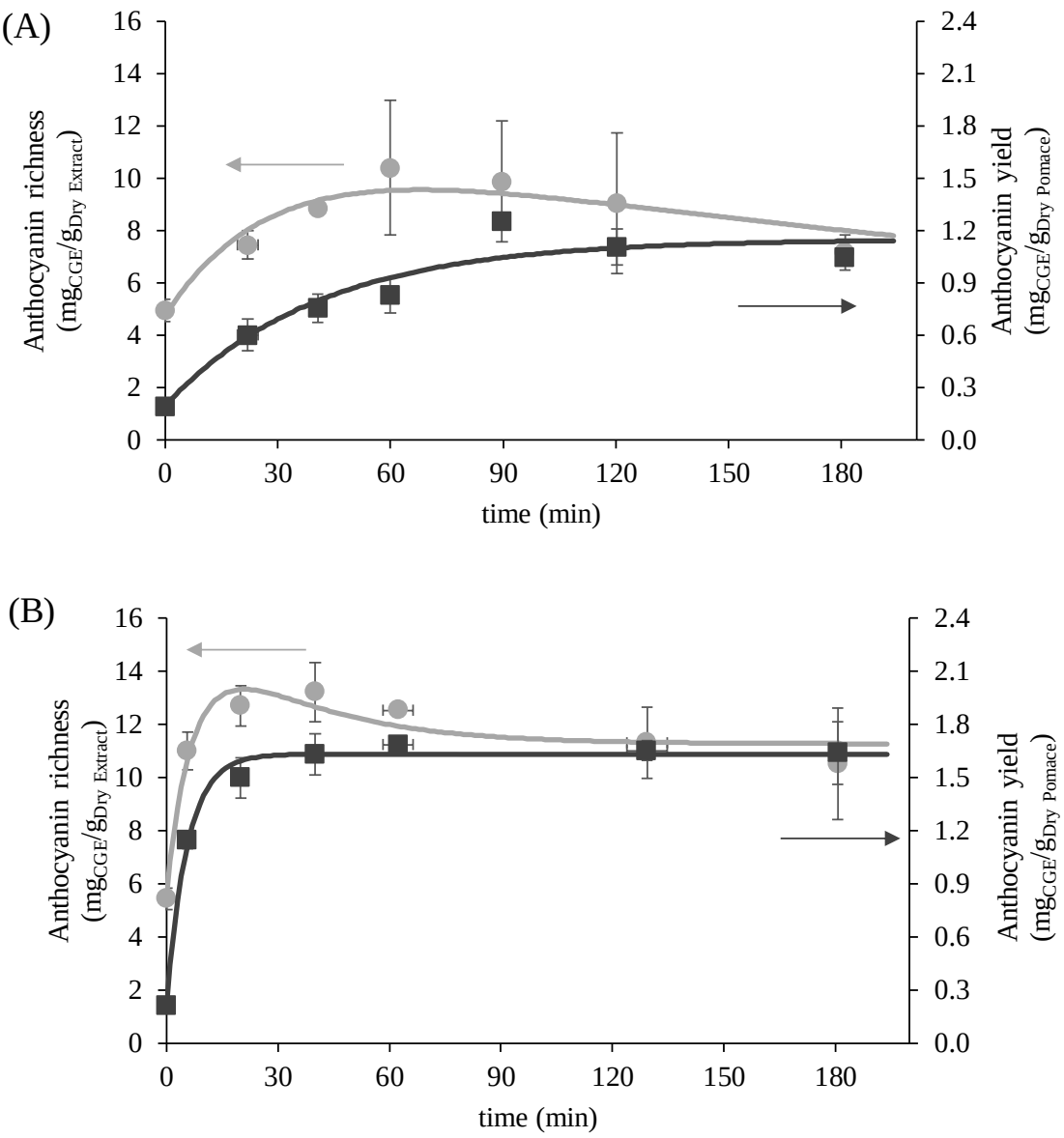

(A)
Anthocyanin richness (mgCGE/gDry Extract)
Anthocyanin yield (mgCGE/gDry Pomace)
time (min)
(B)
Anthocyanin richness (mgCGE/gDry Extract)
Anthocyanin yield (mgCGE/gDry Pomace)
time (min)

Figure 4

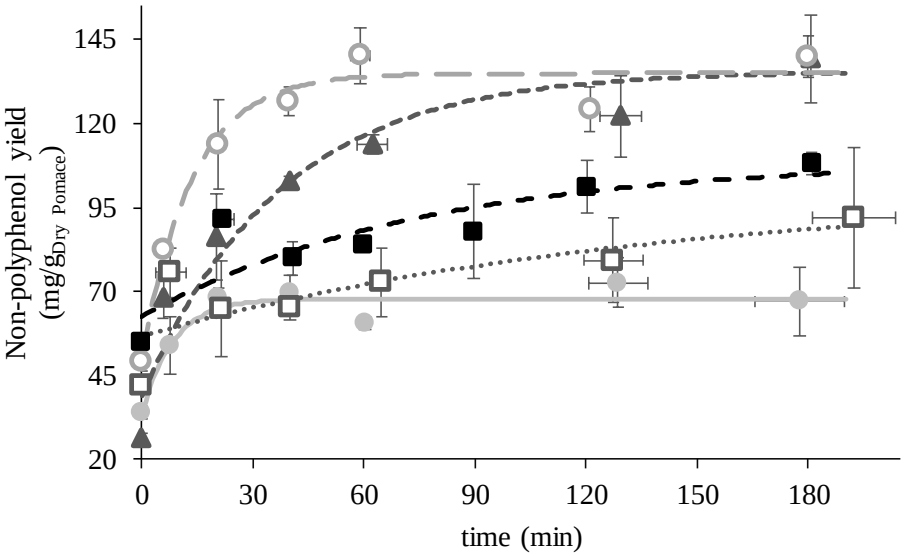

Non-polyphenol yield
(mg/g Dry Pomace)
145
120
95
70
45
20
0
30
60
90
120
150
180
time (min)

Figure 5

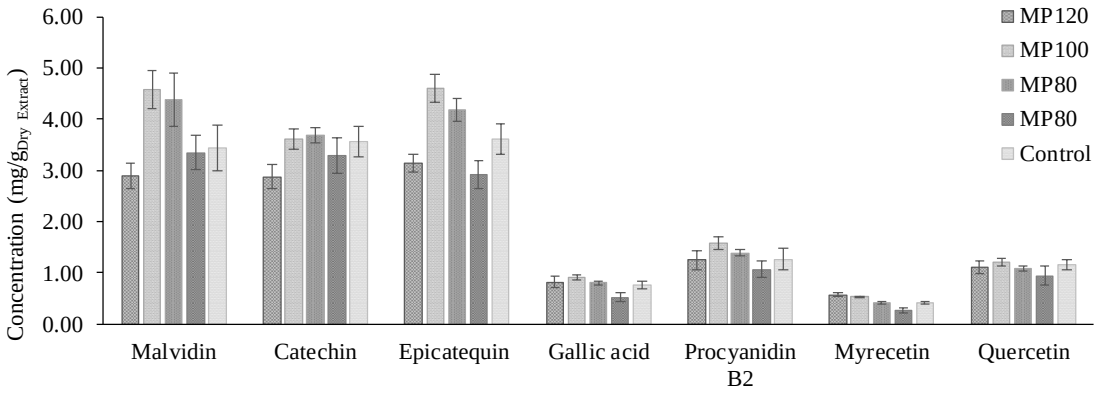

6.00
5.00
4.00
3.00
2.00
1.00
0.00
Concentration (mg/g Dry Extract)
MP120
MP100
MP80
MP80
Control
Malvidin
Catechin
Epicatequin
Gallic acid
Procyanidin B2
Myrecetin
Quercetin

Figure 6

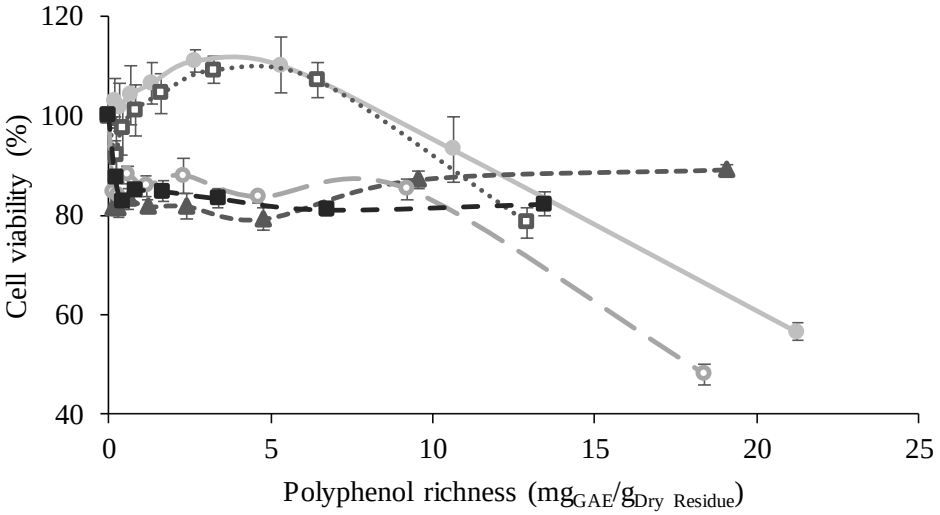
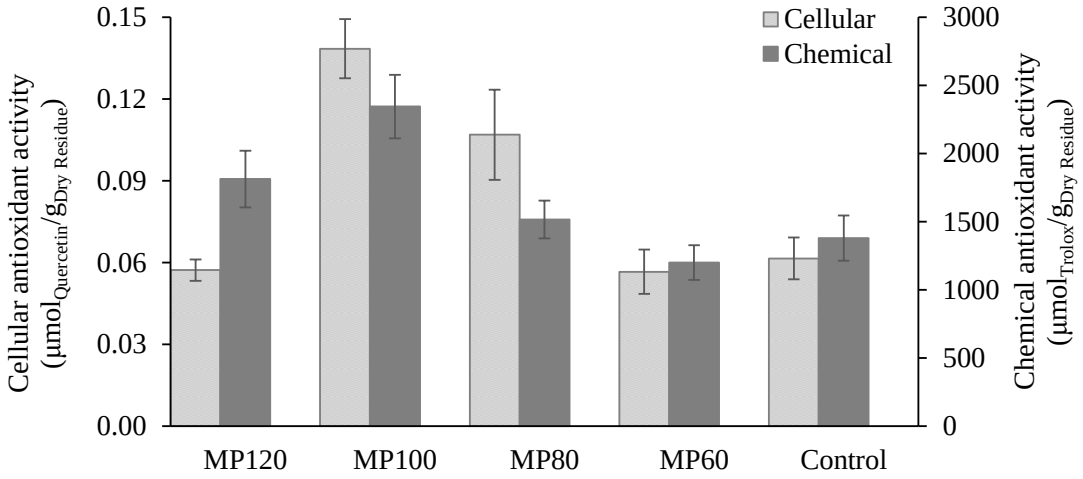

120
100
80
60
40
Cell viability (%)
0
5
10
15
20
25
Polyphenol richness (mg$_{GAE}$/g$_{Dry\ Residue}$)

Figure 7


Cellular
Chemical
Cellular antioxidant activity (μmol$_{Quercetin}$/g$_{Dry\ Residue}$)
Chemical antioxidant activity (μmol$_{Trolox}$/g$_{Dry\ Residue}$)
0.15
0.12
0.09
0.06
0.03
0.00
3000
2500
2000
1500
1000
500
0
MP120
MP100
MP80
MP60
Control

TABLES

Table 1. Ternary mobile phase gradient.

Time (min)	%Eluent A[ai]	% Eluent B[b]	% Eluent C[c]
0	100	0	0
2	100	0	0
5	92	8	0
17	0	14	86
22	0	18	82
29.5	0	21	79
55	0	33	67
70	0	50	50
75	0	50	50
78	20	80	0
81	20	80	0
86	100	0	0

[a]Eluent A: $NH_4H_2PO_4$ 50 mM, pH=2.6

[b]Eluent B: 20% eluent A and 80% acetonitrile

[c]Eluent A: H_3PO_4 200 mM, pH=1.5

Table 2. Extraction kinetic parameters for equation 1.

		YIELD ($mg/g_{DryPomace}$)					RICHNESS (mg/L)				
		MWP120	MWP100	MW80	MW60	Control	MWP120	MWP100	MW80	MW60	Control
Anthocyanins	C_0[d]	2.4	4.4	2.3	2.0	2.1	640	1129	706	820	520
	C_f[e]	21.8	22.0	20.0	25.8	15.8	3.62E+03	3.58E+03	3.19E+03	3.97E+03	4.13E+03
	k[f]	2.47E-01	5.50E-01	1.15E-01	2.91E-02	3.51E-02	1.98E-01	2.66E-01	9.80E-02	2.72E-02	1.43E-02
	ARD[g]	4.0E-02	3.9E-02	7.4E-02	9.2E-02	3.1E-02	4.8E-02	5.6E-02	9.1E-02	1.2E-01	7.1E-02
Polyphenols	C_0	0.2	0.2	0.2	0.2	0.2	61	61	70	55	69
	C_f	0.8	0.7	1.4	0.9	1.0	114	111	224	142	118
	k	2.47E+00	6.24E-01	1.80E-01	1.58E-01	2.45E-02	3.32E-01	2.28E-01	9.26E-02	1.61E-01	2.95E-02
	ARD	6.5E-02	1.3E-01	1.9E-02	5.3E-02	6.5E-02	6.9E-02	1.2E-01	7.8E-02	5.2E-02	6.5E-02
Non-polyphenols	C_0	33.8	56.8	39.0	50.8	62.7	1.32E+04	1.11E+04	1.19E+04	1.24E+04	1.33E+04
	C_f	34.1	44.1	96.7	84.2	46.4	8.47E+03	2.97E+05	1.06E+04	1.09E+04	5.38E+05
	k	1.18E-01	7.11E-03	2.70E-02	7.30E-02	1.34E-02	1.55E-02	8.73E-05	2.72E-02	5.42E-02	5.25E-05
	ARD	4.1E-02	1.0E-01	1.3E-01	4.2E-02	7.7E-02	5.9E-01	6.1E-02	3.5E-01	4.3E-02	8.6E-02
Sugars	C_0	0.3	1.7	1.6	1.1	1.7	67	429	413	301	606
	C_f	11.7	11.5	9.7	13.1	26.8	2.08E+03	1.86E+03	1.62E+03	2.07E+03	9.96E+03
	k	2.33E-01	1.88E-01	1.69E-01	4.29E-02	5.28E-03	2.01E-01	1.79E-01	1.40E-01	4.25E-02	1.50E-03
	ARD	7.5E-02	1.3E-01	6.1E-02	9.4E-02	1.1E-01	8.6E-02	1.3E-01	7.5E-02	8.0E-02	1.4E-01
Fibres	C_0	3.8	3.6	3.2	7.2	7.8	1.01E+03	9.33E+02	9.59E+02	1.44E+03	1.76E+03
	C_f	27.1	21.8	21.9	23.3	34.7	4.32E+03	3.53E+03	3.52E+03	3.76E+03	9.34E+03
	k	4.69E-01	6.74E-02	7.86E-02	2.27E-02	6.09E-03	3.92E-01	5.71E-02	6.23E-02	2.20E-02	2.91E-03
	ARD	2.3E-07	7.5E-09	6.7E-12	1.1E-07	1.8E-09	1.2E-08	8.6E-09	2.2E-09	6.6E-08	2.2E-08

[d] Initial concentration.

[e] Pre-exponential factor.

[f] Rate extraction constant.

[g] Average relative deviation.

Table 3. Representative samples selected from each extraction process.

Extraction process	time (min)	Polyphenols ($mg_{GAE}/g_{Dry\ Extract}$)	Anthocyanins ($mg_{CGE}/g_{Dry\ Extract}$)	Non-polyphenol ($mg_{Dry\ Extrac}/L_{Extract}$)
MP120	8	186.6 ± 6.5	8.3 ± 0.2	1.92E+04 ± 2.33E+03
MP100	21	241.6 ± 6.8	11.2 ± 0.3	1.58E+04 ± 2.43E+03
MP80	40	165.5 ± 5.0	14.0 ± 0.3	2.14E+04 ± 3.91E+02
MP60	21	115.5 ± 4.4	8.7 ± 0.1	2.22E+04 ± 2.47E+03
Control	60	190.4 ± 9.4	10.8 ± 0.4	1.52E+04 ± 5.18E+03