Development of a GreenUltrasound-Assisted Processfor the Preparation of Antioxidant and Pigment-Enriched Extracts from Winery Solid WastesUsing Box-Behnken Experimental Design and Kinetics

Despena Trasanidou[1], Alexandros Apostolakis[2], Dimitris P. Makris[1,✉]

[1]School of Environment, University of the Aegean, Mitr. Ioakim Street, Myrina – 81400, Lemnos, GREECE.

[2]Food Quality & Chemistry of Natural Products, Mediterranean Agronomic Institute of Chania (M. A. I. Ch.),International Centre for Advanced Mediterranean Agronomic Studies (CIHEAM), P.O.Box 85, Chania– 73100, GREECE.

✉ Corresponding author: dmakris@aegean.gr; +30 22540 83114.

Running head title: Green extraction of winery waste polyphenols

Abstract

Red grape pomace, an abundant wine industry solid waste, was used as raw material for the recovery of polyphenols and anthocyanin pigments, using ultrasound-assisted solid-liquid extraction and an extraction medium free from organic solvents, composed of water and glycerol. Glycerol concentration (C_{gl}) and liquid-to-solid ratio ($\mathrm{R}_{\mathrm{L/S}}$) were first optimised employing Box-Behnken experimental design and then extraction was further examined through kinetics.The optimal conditions were found to beC_{gl} = 90% (w/v) and $\mathrm{R}_{\mathrm{L/S}}$ = 90 mL g^{-1}, and under these conditions the extraction of total polyphenols and total pigments was shown to obey first-order kinetics. Maximal effective diffusion (D_{e}) values were 4.22×10^{-12} and 12.59×10^{-12} $\mathrm{m}^2\ \mathrm{s}^{-1}$, for total polyphenols and total pigments, respectively, while the corresponding activation energies (E_{a}) were 13.94 and 8.22 kJ mol^{-1}. Temperature positively affected the antioxidant activity and the extract obtained at 80 °C displayed reducing power of 368.8 μmol ascorbic acid equivalents per g of dry pomace weight.

Keywords:Antioxidants; Box-Behnken design; glycerol; kinetics; pigments; polyphenols; red grape pomace; winery waste

Nomenclature

AED, acoustic energy density (W L^{-1})
C_{gl}, glycerol concentration (%, w/v)
C_{TP}, total polyphenol concentration (mg GAE L^{-1})
D_e, effective diffusion coefficient (m^2 s^{-1})
E_a, activation energy (kJ mol^{-1})
k, extraction rate constant (min^{-1})
k_0, temperature-independent factor (min^{-1})
P_R, reducing power (μmol AAE g^{-1})
R, universal gas constant (J K^{-1} mol^{-1})
r, particle radius (m)
$R_{L/S}$, liquid-to-solid ratio (mL g^{-1})
t, time (min)
T, temperature (°C or K)
Y_{TP}, yield in total polyphenols (mg GAE g^{-1})
Y_{TPm}, yield in total pigments (mg MvE g^{-1})
$Y_{TP(s)}$, yield in total polyphenols at saturation (mg GAE g^{-1})
$Y_{TPm(s)}$, yield in total pigments at saturation (mg MvE g^{-1})

Greek letters

ε, molar absorptivity ($M^{-1}cm^{-1}$)

Abbreviations

AAE, ascorbic acid equivalents
GAE, gallic acid equivalents
MvE, malvidin 3-*O*-glucoside equivalents
MW, molecular weight
RGP, red grape pomace
TP, total polyphenols
TPm, total pigments
TPTZ, 2,4,6-tripyridyl-*s*-triazine
Y, yield

Introduction

An enormous amount of biomass, which mounts up to the sum of billion metric tonnes, is generated on an annual basis from the agricultural industry worldwide. This biomass includes liquid and solid residues and may be considered one of the most abundant, cheap and renewable resources [1]. Agri-food wastes and by-products, if not managed properly, can cause severe environmental risks; hence their efficient valorisation for the production of value-added commodities is of undisputed importance towards the development of sustainable and cleaner processes. In this line, economically viable and environmentally rational strategies are increasingly adopted by the agri-food industry to ensure full exploitation of the residual materials and implement “zero waste” policies.

Wine production is a significant sector of the agricultural economy for many countries around the globe and the winemaking process involves discarding of large amounts of solid residues, such as pomace, stalks and lees. Compared with other abundant similar waste materials, red grape pomace (RGP) contains a particularly high burden of valuable substances, namely polyphenols [2], which include the red, water-soluble, anthocyanin pigments. Polyphenols and pigments are high value-added substances, as they possess variable bioactivities [3, 4], but they are also technologically important as food pigments and antioxidant/antimicrobial preservatives [5].

Thus RGP, owed to its abundance, may be regarded as a prime source of polyphenolic antioxidants and pigments and for this reason numerous investigations have been carried out for the efficient and cost-effective recovery of the aforementioned constituents. The method of preference is solid-liquid extraction, deployed usually following a drying step of the raw material, yet the significance of a wide spectrum of these studies would not go beyond laboratory-scale level. This is because the solvents tested to achieve high recovery yields are toxic and/or highly flammable (methanol, acetone, ethyl acetate), or expensive due to restrictions arising from State laws (ethanol) and therefore completely incompatible with a prospect industrial, “green” extraction process.

Complete removal of these solvents from extracts destined for food, cosmetic or pharmaceutical formulations would inevitably raise issues pertaining to strict quality control, recycling and appropriate safe handling, with an increased associated cost. Hence the search for inexpensive extraction media for the recovery of polyphenolic phytochemicals should embrace methodologies in the direction of ascertaining production of novel formulations without further generation of waste. In this line, the use of low-cost, non-toxic solvent systems for the recovery of target compounds becomes imminent.

Recently, there has been a study reporting on the efficiency of water/glycerol mixtures to extract polyphenolic components from plant material [6, 7]. It was shown that incorporation of relatively low amounts of glycerol (10%, w/v) into water, in combination with moderately high temperature (70 - 80 °C), enabled very satisfactory recovery yields. This was attributed to the low dielectric constant of glycerol, which could lower water's polarity, thus facilitating the extraction of relatively low-polarity molecules, such as polyphenols. On such a ground, the investigation presented herein aimed at optimising polyphenol extraction from RGP, using water/glycerol mixtures, with the view of developing a "green" procedure, free from organic solvents, e.g. methanol or ethanol. The process was developed on the basis of an ultrasound-assisted extraction technique, by a two-step procedure; first, optimisation of critical parameters including glycerol concentration and liquid-to-solid ratio by deploying a Box-Behnken experimental design; and second, critical assessment of the effect of temperature, through kinetics.

Materials and methods

Chemicals and reagents

Ferric chloride hexahydrate was from Acros Organics (New Jersey, U.S.A.). Gallic acid, ascorbic acid, Folin-Ciocalteu reagent and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Sigma-Aldrich (Steinheim, Germany). Glycerol and absolute ethanol were from Fisher Scientific (New Jersey, U.S.A.).

Red grape pomace (RGP)

Pomace originating from vinification of Agiorgitiko variety (*Vitisvinifera* spp.) was kindly provided by the Department of Food Science & Human Nutrition, Agricultural University of Athens. The pomace was dried in an oven at 65 °C for 48 h and then pulverized into a fine powder in a laboratory mill (approximate mean particle size 0.3 mm). The pulverized material was kept at – 20 °C until used.

Batch ultrasound-assisted extraction procedure for the response surface assay

For the Box-Behnken experimental design, an appropriate amount of RGP was mixed with 5 mL aqueous glycerol of defined concentration (**Table 1**), in a 15-mL plastic tube. The mixture was vortexed for a few seconds to form slurry and then subjected to extraction in a temperature-controlled, sonication bath (Elma P70, Singer, Germany), at a fixed power of 140 W, a frequency of 37 kHz, and an acoustic energy density (AED) of 35 W L^{-1}, for 60 min. All extractions were carried out at 45 °C.

Batch ultrasound-assisted extraction procedure for the kinetic assay

Extractions were carried out in plastic containers, using 100 mL of 90% (w/v) aqueous glycerol and an RGP amount to provide a liquid-to-solid ratio of 90 mL g^{-1}. Ultrasound-assisted extractions were performed as above, at 50, 60 and 80 °C. Sampling was accomplished at predetermined intervals. Samples were placed in 1.5-mL Eppendorf tubes and centrifuged in a table centrifugator (Hermle, Wehingen, Germany) at 10,000 rpm for 10 min. The clear solution was used for further analysis.

Determination of total polyphenol yield (Y_{TP})

A previously described protocol was used [7]. Briefly, 0.78 mL of distilled water, 0.02 mL of sample and 0.05 mL of Folin-Ciocalteu reagent were added and vortexed. After exactly 1 min, 0.15 mL of aqueous sodium carbonate 20% was added, and the mixture was vortexed and allowed to stand at room temperature in the dark, for 60 min. The absorbance was read at 750 nm in a Rayleigh 7220G spectrophotometer (Beijing, P.R. China), and the total polyphenol concentration (C_{TP}) was calculated from a calibration curve, using gallic acid as a standard. Yield in total polyphenols (Y_{TP}) was determined as mg gallic acid equivalents (GAE) per g of dry weight (dw), using the following equation:

$$Y_{TP}\ (mg\ GAE\ g^{-1}\ dw) = \frac{C_{TP} \times V}{m} \qquad (1)$$

Where *V* is the volume of the extraction medium (L) and *m* the dry weight of RGP (g).

Determination of total pigment yield (Y_{TPm})

A previously reported methodology was employed [8]. Briefly, 0.1 mL of sample was mixed with 0.9 mL ofHCl solution (0.25 M in ethanol) and the mixture was left to equilibrate for 10 min. The absorbance at 520 nm (A_{520}) was obtained with 0.25 M HCl in ethanol as blank and the total pigment yield was determined as mg malvidin 3-*O*-glucoside equivalents (MvE) per g of dry RGP weight, using as ε = 28,000 and MW = 529 [9], as follows:

$$Y_{TPm}\ (mg\ MvE\ g^{-1}\ dw) = \frac{18.9 \times A_{520} \times V \times F_D}{m} \quad (2)$$

Where *V* is the volume of the extraction medium (L), *m* the dry weight of RGP (g) and F_D the dilution factor.

Determination of the reducing power (P_R)

Determinations were performed according to a previously established protocol [7]. Sample (0.05 mL) was mixed thoroughly with 0.05 mL $FeCl_3$ solution (4 mM in 0.05 M HCl), and incubated for 30 min in a water bath at 37 °C. Following this, 0.9 mL TPTZ solution (1 mM in 0.05 M HCl) was added, and the absorbance was recorded at 620 nm after exactly 5 min. P_R was determined as μmol ascorbic acid equivalents (μmol AAE) per g of dry weight.

Box-Behnken experimental design and response surface methodology

A Box-Behnken experimental design was implemented to determine the optimal extraction conditions for obtainingthehighestextractionyieldin polyphenols and pigments. The independentvariableschosen were liquid-to-solid ratio ($R_{L/S}$) and glycerol concentration (C_{gl}). The two independent variables were coded at three levels, -1, 0 and 1 (**Table 1**), according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \ x_i = 1, 2 \quad (3)$$

Where x_i and X_i are the dimensionless and the actual value of the independent variable i, X_0 the actual value of the independent variable i at the central point and ΔX_i the step change of X_i corresponding to a unit variation of the dimensionless value. Responses (Y_{TP}, Y_{TPm}) at each design point were recorded (**Table 2**).

The data obtained were subjected to regression analysis using least square methodology, to extract the equations that provided the response values as a function of the independent variables (mathematical model). Analysis of variance (ANOVA) was used to assess the statistical significance of the model. Insignificant dependent terms ($p > 0.05$) were omittedfrom the models obtained, through a “backward elimination” process. Contour plots were obtained using the fitted model, by maintaining the independent variables simultaneous.

Statistical analysis and extraction kinetics

Extractions were repeated twice and all determinations were carried out in triplicate. The values obtained were averaged. Box-Behnken experimental design and response surface statistics were performed with JMP™ 10. Kinetics was established by non-linear regression between Yand *t*. Linear and non-linear regressions were performed with SigmaPlot™ 12.0, at least at a 95% significance level.

Results and discussion

Response surface optimisation

A 2-factor,3-level Box-Behnken experimental design was used tooptimize the extractionof polyphenols and pigments from RGP and thus the responses considered were Y_{TP} and Y_{TPm}.Response values as a function of simultaneous variation in both independent variables ($R_{L/S}$ and C_{gl}) were given as contour plots (**Fig. 1**). The ANOVA analysis revealed that for both Y_{TP} and Y_{TPm}, quadratic effects of either $R_{L/S}$ or C_{gl} were not statistically significant. The same held true for cross product terms. Following removal of the non-significant terms, the mathematical models (equations) obtained were those seen in **Table 3**. Values of the independent variables, along with the measured and predicted values for the responses for each point of the experimental design, are analytically presented in **Table 2**. Model fitting was assessed using the square coefficients of correlation (R^2), which for both TP and TPm extractions were ≥ 0.96 ($p< 0.01$), indicating a statistically significant agreement between the observed and predicted responses and that the equations in **Table 3** can reliably predict the experimental results.

The use of the predictive models enabled the theoretical calculation of the optimal set of conditions, which are shown in **Table 4**.It can be seen that for maximising both Y_{TP} and Y_{TPm}, a C_{gl} of 90% (w/v) was required, while regarding optimal $R_{L/S}$, a slight difference was observed. In order to select common optimal conditions for achieving concurrent maximisation of both Y_{TP} and Y_{TPm},the desirability function was utilised. It was found thatan optimumvalue of 0.93 desirability was achieved by setting C_{gl} = 90% (w/v) and $R_{L/S}$ = 90 mL g^{-1} (**Fig. 1**). At those optimal recommended settings, it was predicted that the average maximum Y_{TP} and Y_{TPm}would be11.84±1.09 mg GAE g^{-1}dw and 0.91 ± 0.09 mg MvE g^{-1}dw, respectively.

The determination of the optimal $R_{L/S}$ is of undisputed importance in order to attain the maximum extraction yield and generally the higher the $R_{L/S}$, the higher the yield [10, 11]. Although much lower $R_{L/S}$ have been proposed for the extraction of RGP polyphenols, ranging from 3 mL g^{-1} [12] to 8.7 mL g^{-1} [13], high yields in anthocyanins and polyphenols were obtained using $R_{L/S}$ of 80 – 100 mL g^{-1} [14, 15]. The driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater when a highersolvent-to-solid ratio is used. Whenthe amount of liquid phase compared with that of the dispersed phase is not sufficient to obtain adequate transfer, variousequilibriamay take place, leading to a non-negligible resistance to mass transfer. Therefore it is crucial to have a well-defined$R_{L/S}$ in order to achievesufficient mixing and thus high diffusion rate of the solute during the extraction process [16].

The higher extraction yield seen by increasing C_{gl} may be mostly ascribed to the polarity of glycerol. It has been argued that addition of glycerolto water would favour the solubilisation of relatively low-polarity molecules, such as polyphenols, because of glycerol's lower polarity[7].This is consistent with findings supporting that polyphenols may be easily solubilised in polar protic media, such as hydroethanolic and presumably, hydroglycerolic mixtures, although it hasalso been emphasised that the solubility of phenols in different solvents cannot be based on their polarities;solubility is a complicated phenomenon, governed by other parameters, such asthe stereochemistry of phenols (the polar and the non-polar fragmentson the molecule) and the intermolecular forces (mainly hydrogen bonds) between them and the solvent[17].

Extraction kinetics and the effect of temperature

RGP extractions were performed using the optimised conditions, that isC_{gl}= 90% (w/v) and $R_{L/S}$ = 90 mL g^{-1}.The model best fitted to the extraction kinetics using non-linear regression between Y_{TP} and Y_{TPm} values, and t (**Fig. 2**), was a 2-parameter, single exponential rise-to-maximum, described by the equation:

$$y = a(1 - e^{-bx}) \tag{4}$$

For both Y_{TP} and Y_{TPm} and for all temperatures tested, fitting was high and statistically significant (R^2> 0.97, p< 0.0001).This suggested that extraction yield for TP and TPmas a function of t can be adequately predicted by the eq. (4), which represents first-order kinetics, considering the boundary conditions t = 0 to t and Y_t = 0 to Y_t:

$$Y_t = Y_s(1 - e^{-kt}) \tag{5}$$

Where Y_t is the extraction yield at any time t, Y_s the extraction yield at saturation (equilibrium) and k the apparent first-order extraction rate constant. Both Y_s and $k$values were calculated by non-linear regression, using SigmaPlot™ 12.0.

Rearrangement of eq. (5) would give:

$$ln\left(\frac{Y_s}{Y_s - Y_t}\right) = kt \tag{6}$$

Based on Fick's second law, the mathematical expression that links Y and the effective diffusion of the solute (polyphenols/pigments) can be described as follows [18]:

$$\frac{Y_t}{Y_s} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n}e^{-\frac{D_e n^2 \pi^2 t}{r^2}} \tag{7}$$

Where D_e is the effective diffusion coefficient ($m^2 s^{-1}$), and *r* the radius of the RGP particle (m). However, after the elapse of a short extraction period, only the first term of the series solution is considered significant, hence eq. (7) can be written as:

$$1 - \frac{Y_t}{Y_s} = \frac{6}{\pi^2} e^{-\frac{D_e \pi^2 t}{r^2}} \qquad (8)$$

The linearized form of eq.(8) would be:

$$ln\left(\frac{Y_s}{Y_s - Y_t}\right) = ln\frac{\pi^2}{6} + \frac{D_e \pi^2 t}{r^2} \qquad (9)$$

The D_e coefficient can then be calculated graphically, from the slop of the straight line (slope $=\frac{D_e \pi^2}{r^2}$), obtained after plotting $ln\left(\frac{Y_s}{Y_s - Y_t}\right)$ against *t*.

The above considerations regarding diffusion were admitted, assuming that:

1. Polyphenols and pigments were homogeneously distributedwithin the solid particles.
2. The particles were considered as being spherical.
3. The diffusion coefficient remained constant throughout the extraction process.
4. The solution was perfectly mixed upon the energy dissipated by the ultrasonic waves.
5. Resistance to mass transfer was negligible in the liquid phase.
6. The transport of polyphenols/pigments from the solid particles into the liquid phase occurred through diffusion; diffusion of polyphenols and pigments proceeded simultaneously without interactionsbetween them.

On such a theoretic basis, the kinetic parameters deriving from engineering the extraction process for both TP and TPm, were determined and analytically presented in **Table 5**.Raising the temperature from 50 to 80 °C had a positive effect on both $Y_{TP(s)}$ and $Y_{TPm(s)}$, provoking corresponding increases by 2.63 and 1.14 times. The effect of temperature on the extraction of RGP in several instances is positive [13, 19, 20], since higher temperatures facilitate polyphenol diffusion and increase solubility [17, 21].Furthermore, it has been demonstrated that solubilisation of catechin, an abundant RGP constituent, is endothermic and thus thermodynamically favoured at higher temperatures [22]. Similar phenomena may hold true for other RGP constituents too. On the other hand, temperature cannot be increased beyond certain limits, as this has been proven detrimental to anthocyanins, inducing their thermal degradation [23, 24].

The maximum $Y_{TP(s)}$, achieved at 80 °C, was 66.70 mg GAE g^{-1}dw. This level is much higher than 0.32 mg GAE g^{-1}fw reported for UAE of polyphenols from RGP using water [25] and 7.7mg GAE g^{-1}dw, reported for UAE of polyphenols from RGP using 50% ethanol [26]. However, yields as high as 72.60 mg GAEg^{-1}fw [9], 55.00 mg GAE g^{-1}dw [12] and 31.69 mg GAE g^{-1}dw [19] were achieved with conventional extraction techniques, using 57% ethanol, 66% ethanol and subcritical water, respectively. Likewise, $Y_{TPm(s)}$ at 80 °C was 4.19 mg MvE g^{-1}dw, which is lower than 7.76 mg g^{-1}dw of total anthocyanins found for UAE of RGP with 50% ethanol [27], yet significantly higher than 1.86 mg g^{-1}dwachieved with microwave-assisted extraction of RGP with 50% methanol [28], 1.87 mg g^{-1}dw with 57% ethanol [9], and 1.30 mg g^{-1}dwwith a combination of hot-cold water [29]. Even lower levels of 0.74 mg g^{-1}dw [13] and 0.70 mg g^{-1}dw [12] were obtained when 1.5 M HCl in ethanol and 66% ethanol were employed as the extraction media, respectively, but the use of subcritical water/ethanol mixtures afforded a comparable yield of 4.63 mg g^{-1}dw [24].

Regarding the kinetics of TP extraction, *k* was found to increase in response to raising the temperature, reaching 0.029 min^{-1} at 80 °C. This value is lower than 0.130 min^{-1}reported for polyphenol extraction from grape juice bagasse using 50% ethanol [30]and0.088 min^{-1} reported for UAE of polyphenols from apple peels at 40 °C using water [31], but higher than 0.012 min^{-1} found for water extraction of *Tilia* sapwood polyphenols at 80 °C [32]. By contrast, TPm extraction was faster than TP at any temperature tested, achieving a *k* value of 0.083min^{-1} at 80 °C. This is consistent with the outcome from previous studies on the extraction of anthocyanins from RGP with 50% ethanol, giving *k* values between 0.034 and 0.157 min^{-1}, within a temperature range of 25 – 60 °C [33].

In order to obtain quantitative data pertaining to the effect of temperature on the extraction rate,the Arrhenius equation was used:

$$k = k_0 e^{-\frac{E_a}{RT}} \qquad (10)$$

Where k_0 is the temperature-independent factor (min^{-1}), R the universal gas constant (8.314 J K^{-1} mol^{-1}), T the absolute temperature (K) and E_a the activation energy (J mol^{-1}). Transformation of eq. (10) allows obtaining a linear relationship between the first-order extraction rate constant and the inverse of T:

$$lnk = lnk_0 + (-\frac{E_a}{R})\frac{1}{T} \qquad (11)$$

Therefore,E_a could be determined graphically, since the straight line obtained by plotting lnk as a function of $1/T$ would have a slope $=-\frac{E_a}{R}$.

The E_a determined for TP extraction was 13.94 kJ mol^{-1}.This value is almost 3 times higher than 4.6 kJ mol^{-1}, determined for theUAE of TP from RGP [26], employing 50% ethanol and almost 2.2 times higher than 6.34 kJ mol^{-1} found for the UAE of orange peel polyphenols with 75% ethanol [34]. It should be emphasised that E_a determined for the extraction of TP from various plant sources varied from 0.5 kJ mol^{-1}[21] to as high as 97.1 kJ mol^{-1} [14], depending on the solvent system and the conditions used. In general, the sufficient amount of$E_a$for polyphenol extraction lies from 14.54 [35]to 56.00kJ mol^{-1}[18]. By contrast, theE_a required for TPm extraction was found to be8.22 kJ mol^{-1},a very low level compared with 76.7 kJ mol^{-1} reported for anthocyanin extraction from milled berries, using 67% ethanol [36] and significantly lower than 29.5 kJ mol^{-1} estimated for anthocyanin extraction from RGP, using 50% ethanol [33].

In both cases $E_a$were positive, which is in agreement with endothermic process. E_a may be associated with both medium and matrix resistance, which the solute should overcome. If $E_a < 20$ kJ mol^{-1}, then extraction is managed by diffusion [26]. Ultrasounds can assist with extraction processes both through cell disruption and by enhancing mass transfer in the boundary layer surrounding the solid matrix [37]. The relatively low E_a levels found for both TP and TPm extraction indicated that ultrasonication is an effective means of assisting extraction, by providing the appropriate energy dissipation for efficient mass transfer. The ultrasonic energy is thought to accelerate the diffusional process by enhancing the solid particle permeability by the solvent, hence facilitating polyphenol release [38].It could also be argued that the lower energy barrier required to initiate diffusion is provided by ultrasonic energy, which may contributein overcoming solute - solute and solute - matrix interactions, thus decreasing E_a of the extraction process.

To support this hypothesis, the effective diffusions (D_e) for both TP and TPm were also calculated (**Table 5**). As can be seen, TP extraction attained a D_e level of 4.22 × 10^{-12} m^2 s^{-1}, at 80 °C, which is higher than 0.14 – 1.57 × 10^{-12} m^2 s^{-1}reported for extraction of lignans from flaxseed [18]and 1.05 × 10^{-12} m^2 s^{-1} for polyphenols extraction from RGP with 50% ethanol [39], but lower than 12.3 – 15.0 × 10^{-12} m^2 s^{-1} achieved in the extraction of polyphenols from RGP using 60% ethanol [40].D_e as high as 123× 10^{-12} m^2 $s^{-1}$and 12 – 250 × 10^{-12} m^2 s^{-1}were also determined for polyphenol and anthocyanin extraction from milled berries with 67% ethanol, respectively [36]. Diffusion of TPm was faster at 80 °C, reaching 12.59 × 10^{-12} m^2 s^{-1}, which is in consistency with the above-mentioned data.

Reducing power (P_R)

P_R is a reliable criterion of antioxidant activity and it has been demonstrated that there is a statistically significant correlation between the amount of polyphenols and P_R, but also between $P_R$and radical scavenging for various polyphenol-containing materials [2]. The determination of P_R following 60 min of extraction provided a clear picture regarding the antioxidant activity of the extracts obtained (**Fig. 3**), which was found to increase by approximately 2.4-times, upon increasing the extraction temperature from 50 to 80 °C. This is particularly important, indicating that risingthe extraction temperature up to 80 °C does not provoke any loss of antioxidants and that the higher Y_{TP} is presumably accompanied by a proportional antioxidant effect. It should be stressed that the proportionality between the polyphenolic content and the antioxidant activity is not a general principle, as demonstrated by previous examinations [41 – 43]. Although higher polyphenol levels are usually accompanied by higher P_R, the utilisation of increased extraction temperatures might compromise radical scavenging [35]. On the other hand, the expression of antioxidant effects of a mixture should be interpreted with caution, because the lack of proportionality might be a consequence of antagonism [44].

Conclusions

This study demonstrated for the first time that an extraction medium composed of 90% (w/v) aqueous glycerol can efficiently extract polyphenols and pigments from red grape pomace, with the assistance of ultrasonication. Extraction yield was found to increase in response to raising the temperature up to 80 °C, a phenomenon attributed to increased diffusion. The relatively low activation energies for the extraction of total polyphenols and total pigments were ascribed to the effect of ultrasounds, which were hypothesised to provide the appropriate dissipation energy for such a process. The satisfactory extraction yields achieved were in concurrence to this theory. This is of utmost importance, considering that glycerol is an inexpensive, abundant and non-toxic bio-material. Hence the adoption of similar processes by the industries would be expected to form the basis for the development of green procedures, aimed at the valorisation of food industry waste streams and the sustainable production of value-added commodities, such as food additives, food supplements, pharmaceutical formulations and cosmetics.

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Table 1: Experimental values and coded levels of the independent variables used for the Box-Behnkenexperimental design.

Independent variables	**Code units**	**Coded variable level**		
		-1	**0**	**1**
$R_{L/S}$ (mL g^{-1})	X_1	10	50	90
C_{gl} (%, w/v)	X_2	10	50	90

Table 2: Measured and predicted Y_{TP} andY_{TPm} values, determined for the individual points of the experimental design.Extractions were carried out under sonication (140 W, 37 kHz, 35 W L^{-1}), at 45 °C, for 60 min.

Design point	**Independent variables**		**Responses**			
	$R_{L/S}$ (X_1)	**C_{gl} (X_2)**	**Y_{TP} (mg GAE g^{-1}dw)**		**Y_{TPm} (mg MvE g^{-1}dw)**	
			Measured	**Predicted**	**Measured**	**Predicted**
1	10 (-1)	10 (-1)	2.25	2.20	0.06	0.04
2	10 (-1)	90 (1)	8.09	8.30	0.48	0.51
3	90 (1)	10 (-1)	4.32	4.06	0.12	0.13
4	90 (1)	90 (1)	11.85	11.84	0.84	0.90
5	10 (-1)	50 (0)	4.95	4.79	0.18	0.17
6	90 (1)	50 (0)	7.22	7.49	0.49	0.41
7	50 (0)	10 (-1)	3.43	3.73	0.21	0.22
8	50 (0)	90 (1)	10.86	10.66	0.94	0.84
9	50 (0)	50 (0)	7.25	6.74	0.43	0.42
10	50 (0)	50 (0)	6.32	6.74	0.33	0.42

Table 3: Equations (mathematical models) and statistical parameters describing the effect of the independent variables ($R_{L/S}$ and C_{gl}) on the extraction of polyphenols and pigments, calculated after implementation of a Box-Behnken experimental design.

Response variables	**Polynomial equations**	**R^2**	***p***
Y_{TP} (mg GAE g^{-1}dw)	$0.710 + 0.034R_{L/S} + 0.087C_{gl}$	0.99	0.0003
Y_{TPm} (mg MvE g^{-1}dw)	$– 0.118 + 0.003R_{L/S} + 0.078C_{gl}$	0.96	0.0066

Table 4: Optimal predicted conditions and maximal predicted values for the extractionof polyphenols and pigments from RGP.Extractions were carried out under sonication (140 W, 37 kHz, 35 W L^{-1}), at 45 °C, for 60 min.

Response	**Maximal predicted value**	**Optimal conditions**	
		C_{gl} (w/v, %)	**$R_{L/S}$ (mL g^{-1})**
Y_{TP} (mg GAE g^{-1}dw)	11.84±1.09	90	90
Y_{TPm} (mgMvE g^{-1}dw)	0.91±0.09	90	79

Table 5: Kinetic parameters determined for the extraction of polyphenols and pigments from RGP, using 90% (w/v) aqueous glycerol. Extractions were carried out at $R_{L/S}$ = 90 mL g^{-1}, under sonication (140 W, 37 kHz, 35 W L^{-1}).

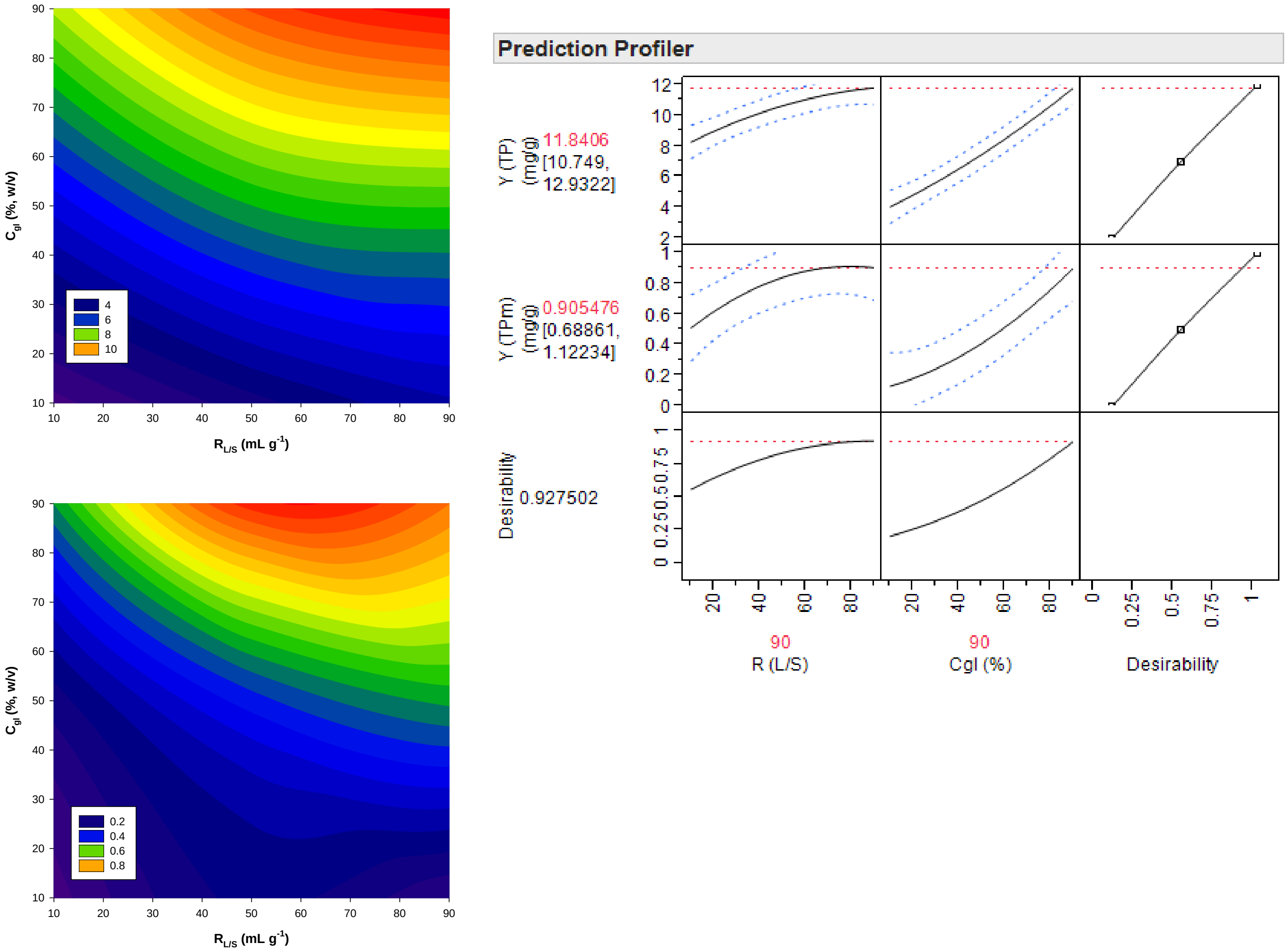
Kinetic parameters	**Temperature (°C)**		
	50	**60**	**80**
Total polyphenols			
k (min^{-1})	0.019	0.021	0.029
D_e (m^2s^{-1}) × 10^{-12}	2.73	3.07	4.22
$Y_{TP(s)}$ (mg GAE g^{-1})	25.36	29.25	66.70
Total pigments			
k(min^{-1})	0.063	0.078	0.083
D_e (m^2s^{-1}) × 10^{-12}	9.59	11.89	12.59
$Y_{TPm(s)}$ (mg MvE g^{-1})	3.68	3.86	4.19

FIGURE CAPTIONS

Fig. 1: Left: Contour plots illustrating the effect of simultaneous variation of $R_{L/S}$ and C_{gl} on the Y_{TP} (upper plot) and Y_{TPm} (lower plot). Values in the frames are expressed in mg GAE g^{-1} dw and mg MvE g^{-1} dw, for $Y_{TP}$and Y_{TPm}, respectively.Right: Prediction profiler displaying the overall desirability of the model, after fixing $R_{L/S}$ = 90 mL g^{-1} and and C_{gl} = 90% (w/v). Extractions of RGP were carried out under sonication (140 W, 37 kHz, 35 W L^{-1}), at 45 °C, for 60 min.

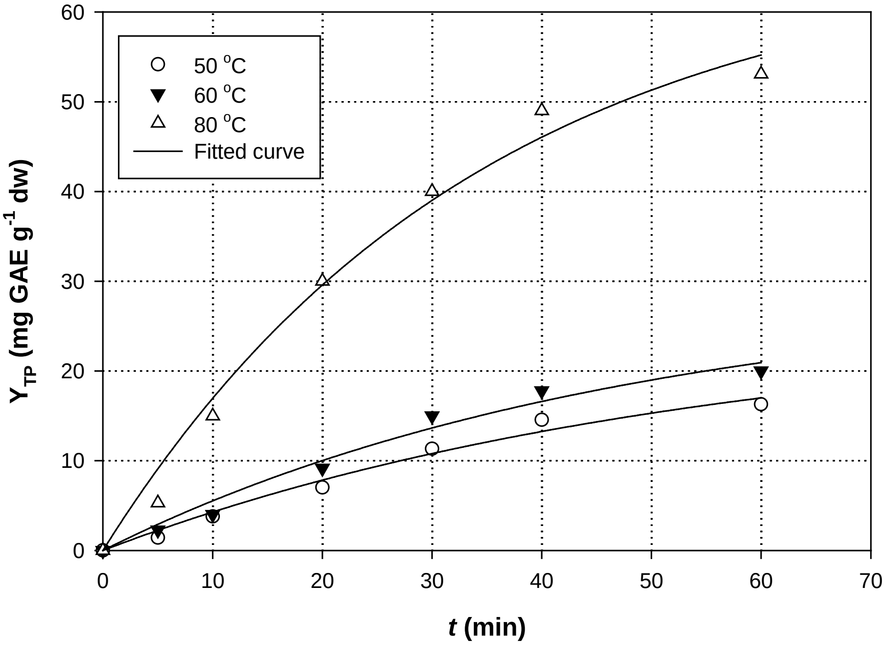
Fig. 2:Non-linear regression between Y and *t* values during extraction of TP (upper plot) and TPm(lower plot) from RGP; $R_{L/S}$ = 90 mL g^{-1} and C_{gl} = 90% (w/v). Extractions were carried out under sonication (140 W, 37 kHz, 35 W L^{-1}).

Fig. 3: P_R evolution of RGP extracts, upon increasing extraction temperature. Values reported were determined in extracts obtained with $R_{L/S}$ = 90 mL g^{-1} and C_{gl} = 90% (w/v), under sonication (140 W, 37 kHz, 35 W L^{-1}), after 60 min.

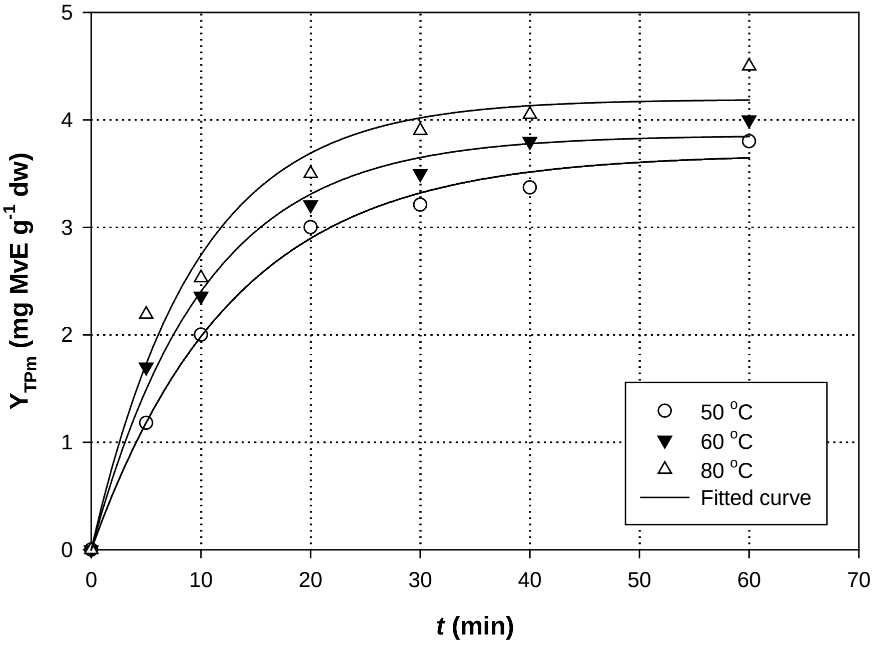


C_{gl} (%, w/v)
$R_{L/S}$ (mL g^{-1})
4
6
8
10
0.2
0.4
0.6
0.8
Prediction Profiler
Y (TP) (mg/g)
11.8406
[10.749, 12.9322]
Y (TPm) (mg/g)
0.905476
[0.68861, 1.12234]
Desirability
0.927502
90
R (L/S)
90
Cgl (%)
Desirability

Fig. 1



50 °C
60 °C
80 °C
Fitted curve
Y_{TP} (mg GAE g^{-1} dw)
t (min)
0
10
20
30
40
50
60
70



50 °C
60 °C
80 °C
Fitted curve
Y_{TPm} (mg MvE g^{-1} dw)
t (min)
0
1
2
3
4
5
10
20
30
40
50
60
70

Fig. 2

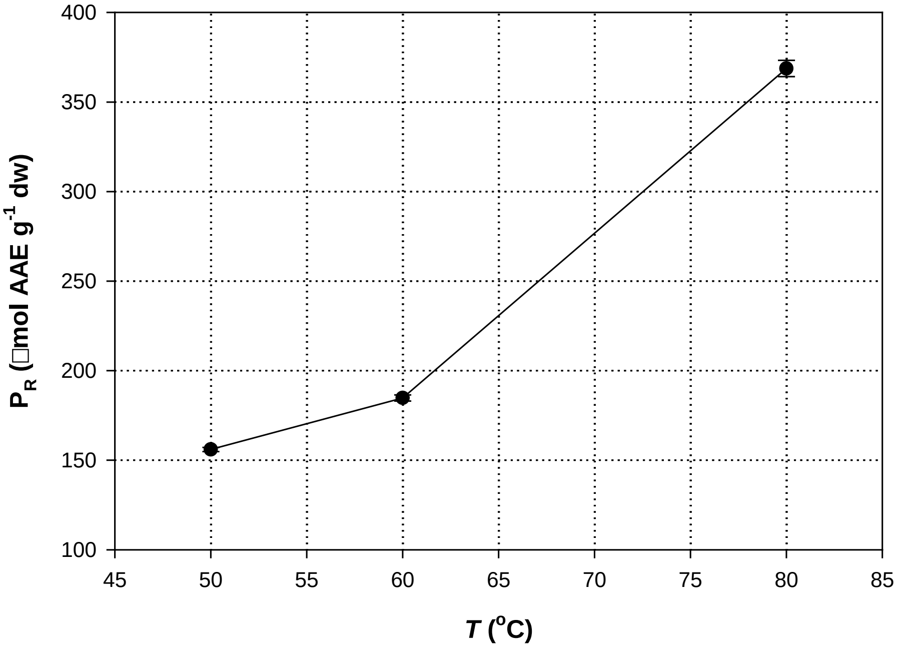

400
350
300
250
200
150
100
45
50
55
60
65
70
75
80
85
P_R (□mol AAE g^-1 dw)
T (°C)

Fig. 3