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Dynamic superheated liquid extraction of anthocyanins and other phenolics from red grape skins of winemaking residues

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Abstract

Grape skins from a grape pomace were subject to extraction with superheated ethanol–water mixtures for quantitative extraction of anthocyans and other phenolic compounds. The variables affecting dynamic extraction of these compounds were studied and identification and quantification of the extracted compounds were performed by both direct spectrophotometry or after HPLC separation using UV or MS detectors. The optimal working conditions for total extraction of anthocyans were: 1:1 (v/v) ethanol–water acidified with 0.8% (v/v) HCl, 120 °C, 30 min, 1.2 ml/min and 80 bar. The yields of anthocyanins, total phenolics and flavanols thus obtained were much higher (3 times for anthocyanins, 7 times for total phenolics and 11 times for flavanols) than those provided by dynamic conventional solid–liquid extraction. Several sample preparation procedures for skins as alternatives to free-drying were also investigated and drying at 40 °C for 24 h provided the best results. Extraction with acidified water provides similar composition and poorer efficiency than 1:1 ethanol–water; also similar to two commercial grape skin extracts used as natural colorants.
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1. Introduction

Grape skin contains a great number of polyphenolic compounds, the concentration of which varies greatly according to the variety of grapevine and is influenced by cultivar, season and environmental factors (Ojeda et al., 2002). The most abundant of these compounds in red grapes are anthocyans, mainly 3-glycosides, 3-acetylglycosides and 3-*p*-coumaroylglycosides of malvidin (Mv), peonidin (Pn), delphinidin (Dp), petunidin (Pt) and cyanidin (Cy) (Wulf and Nagel, 1978), but tartaric esters of hydroxycinnamic acids, monomeric and dimeric flavanols, flavonols and stilbenes are also found (Rodríguez Montealegre et al., 2006).

The potential health benefits of these compounds have been widely reported. They are strong antioxidants and free radical scavengers (Lapidot et al., 1999 and Woodman et al., 2005) with anticarcinogenic (Zhao et al., 1999), angioprotective (Vennat et al., 1988), anti-inflammatory (Pietta et al., 2003) and antibacterial activities (Fukai et al., 1991); thus, they are related with the reduction of risk of coronary heart disease (Hung et al., 2000), circulatory disorders (Bettini et al., 1985), some types of tumours (Sakagami et al., 2005) and chronic diseases (Dryden et al., 2006).

These polyphenols are partially extracted into the must during the winemaking process. In the case of red wines, the crushed grapes are kept in contact with the juice during fermentation for several days in order to enrich it with these compounds (mainly with anthocyanins). Nevertheless, the extraction is far for being complete – e.g. the yield estimated for anthocyanins is 30–40% (Van Balen, 1984). Therefore, the skins from the remaining solid waste of

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pressed grapes – the grape pomace –, which represent ca. 50% of its weight, constitute a very abundant and inexpensive source of these phenolic compounds.

At present, the most valuable use for this waste is probably the obtainment of anthocyanin pigments, which are of interest for the food, cosmetic and pharmaceutical industries to be used as substitutes for synthetic colorants and antioxidants (Giusti and Wrolstad, 2003). The most usual industrial methods for obtaining these pigments are based on conventional solid–liquid extraction with hydroalcoholic mixtures – ethanol is preferred when the product is for human consumption (Ju and Howard, 2003) – or heated sulphured water (Sefcal process) (Cacace and Mazza, 2002). An extraction process based on adsorptive resins has also been developed (Aplexion process) (Flanzy, 2000).

Superheated liquids can be an attractive industrial alternative for the extraction of these compounds with two fundamental advantages over conventional techniques, namely: (a) Raising the temperature above the boiling point of the solvent increases the diffusion rate, solubility and mass transfer of the compounds and decreases the viscosity and surface tension of the solvent. These changes improve the contact of the compounds with the solvent and enhance extraction, which can then be achieved more rapidly and with less solvent consumption as compared with conventional industrial methods. (b) The absence of light and air significantly reduce both degradation and oxidation of these compounds during extraction (Escribano-Bailón and Santos-Buelga, 2003).

In the last years, superheated liquid extraction (SLE) has been successfully applied to the extraction of different phenolic compounds from grapes – e.g. catechins and proanthocyanidins from grape seeds and trans-resveratrol from grapes (Piñeiro et al., 2006). In order to develop methods for the analysis of these biological samples, SLE has been employed as the first step of the process using methanol as extractant. Ju and Howard (2005) have investigated in a discontinuous mode the effect of different solvents and temperature conditions on the SLE of anthocyanins from fresh grape skins obtained from a highly pigmented advanced breeding line of wine grape. They conclude that either superheated (110 °C) water or sulphured water have a effectiveness similar to that of conventional hot water or 60:40 methanol–water to extract anthocyanins.

Continuous extraction of anthocyanins and other polyphenolic compounds from grape pomace skins by superheated ethanol–water mixtures is present here with a view of subsequent implementation to pilot-plant scale. Two fundamental innovative aspects of this study are worth emphasizing: (a) Skins from grape pomace are extracted as such, unlike previous works, where skins from fresh grapes were used. This fact is of paramount importance because skins from grape pomace have already been extracted during the winemaking process, so they only contain strongly retained anthocyanins and other phenolic compounds. Therefore, it is foreseeable that more drastic conditions are required. (b) Dynamic extraction allows supporting contact between the sample and fresh extractant, thus accelerating mass transfer and shortening the extraction time.

2. Methods

2.1. Samples and chemicals

Grape pomace was supplied by Alvinesa (Daimiel, Ciudad Real, Spain). Skins were separated manually, kept at 40 °C for 24 h and then milled up to a particle size lower than 0.42 mm diameter. The powder was kept in a desiccator until use. Ethanol (96%, v/v) PA from Panreac (Barcelona, Spain) and distilled water were used for preparing the different ethanol–water mixtures. Acetonitrile and formic acid (both HPLC grade and supplied by Panreac Barcelona) were used to prepare the mobile phases. Ultra-pure water was obtained from a Millipore (Bedfore, MA, USA) Milli-Q plus system. Mv, Pn, Pt, Cy, Dp and their glycosides, as resveratrol, used as chromatographic standards, were from Extrasynthese (Genay, France). (+)-Catechin, quercetin and caffeic, coumaric and ferulic acids were from Sigma–Aldrich (St. Louis, USA) The nitrogen for dragging the extract from the extraction cell was supplied by Carburos Metálicos (Barcelona, Spain).

2.2. Apparatus and instruments

Skins were milled with a grinder (Moulinex D56, Barcelona, Spain).

Superheated liquid extractions were performed with the approach depicted in Fig. 1, which consists of the following units: (a) an extractant reservoir; (b) a high-pressure pump (Shimadzu LD-AC10) which propels the extractant through the system; (c) a selection valve (V_1) located next to the pump, which allows flushing the extract with dry N_2 after extraction; (d) a coil enables extractant to be heated at working temperature before entering the extraction chamber; (e) a stainless steel cylindrical extraction chamber (200 mm × 10 mm i.d., 15 ml internal volume) where the sample is placed. This chamber is closed at both ends with screws whose caps contain stainless steel filter plates (1 mm thick, 12 mm diameter) to ensure the sample is not carried away by the extractant; (f) a restriction valve (V_2) to maintain the preset pressure in the system; (g) a cooler made of stainless steel tubing (1 m length, 0.4 mm i.d.) and refrigerated with water; (h) a gas chromatograph oven (Konix, Cromatix KNK-2000) where the extraction chamber is placed and heated.

Shaking and centrifugation of the extracts were carried out by means of an MS2 Minishaker (IKA, Germany) Vortex and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

The absorbance of the extracts was measured by an Agilent 8453E UV–visible Spectrometer (Waldbronn, Ger-

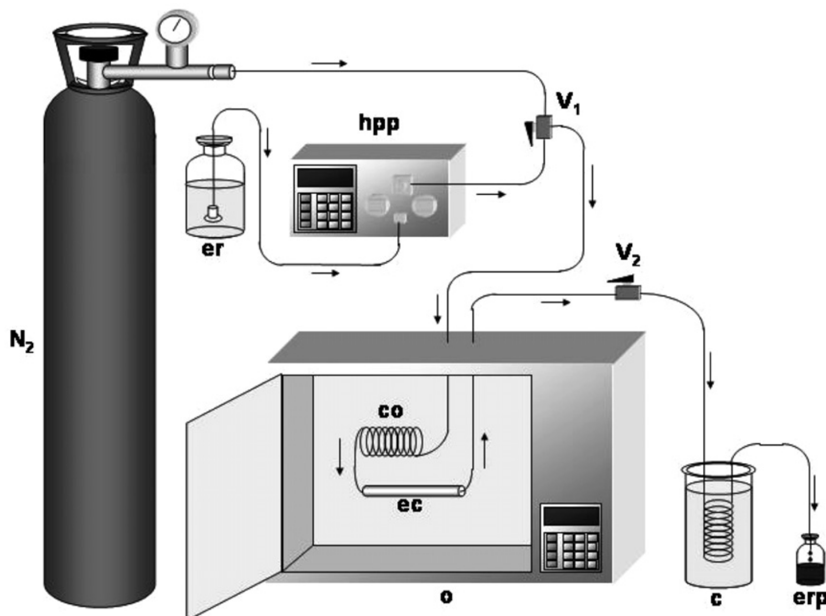

Fig. 1. The extraction system used. er = extractant reservoir, hpp = high-pressure pump, co = coil, ec = extraction cell, o = oven, c = cooler, V_1 = selection valve, V_2 = restriction valve; erp = extract receptacle.

many) and they were analysed by a modular 1100 Hewlett–Packard liquid chromatograph (Pittsburg, PA, USA), consisting of a G1311A high-pressure quaternary pump, a G1322A vacuum degasser, a 7725 Rheodyne high-pressure manual injection valve (HPIV) and a G1315A diode array detector.

HPLC-MS analysis was carried out by an Agilent 1100 Series liquid chromatograph, consisting of degasser, high-pressure quaternary pump, autosampler, column oven, diode array detector and MS-ESI detector (LC/MSD Trap VL).

Statgraphics plus v. 5.1. for Windows was used for the multivariate studies.

2.3. Methods

2.3.1. Superheated ethanol–water extraction (SEWE)

Milled skins (1–3 g) were mixed with 3 mm diameter glass balls and placed in the extraction cell and this one into the oven. Then, a flow-rate of 5 ml/min was established to fill the cell quickly (3 min). In order to ensure the absence of air in the system, valve V_2 was open until the first drop of extractant appeared at the end of the cooler. At that moment, valve V_1 was closed, the pump put off and the oven heater turned on. When the selected temperature and pressure values were reached (5 min), the pump was put on again and the required flow-rate was fixed. At the same time, valve V_1 was opened and the restriction valve (V_2) was regulated for keeping the pressure. When the extraction time was over, the oven heater was turned off, the restriction valve was opened completely and valve V_1 was switched to flush out the extract by dry nitrogen.

2.3.2. Determination of total anthocyans

The amount of total anthocyans in the extracts was determined by measuring absorbance at 535 nm against a blank of 1% HCl in distilled water. A calibration curve was run with solutions of 5, 10, 15, 20, 25, 30, 35 and 40 mg of malvidin-3-glycoside (Mv3G)/l in 1% HCl in distilled water ($y = 0.0403x + 0.0227$; $r^2 = 0.9999$). All the extracts were diluted with 1% in HCl distilled water until the absorbance was within the calibration limits. The concentration of anthocyans thus obtained was multiplied by the dilution factor of the extract volume and divided by the weight of skins employed. In this way, the results were expressed as the equivalent to milligrams of Mv3G per gram of skin (mg Mv3GE/g).

2.3.3. Determination of total phenolics

The amount of total phenolics was measured by a modified version of the Folin–Ciocalteu method (Natera, 1981) using gallic acid as standard, for which a calibration curve was run with solutions of 100, 200, 300, 400, 500 and 600 mg/l of this compound ($y = 0.0025x + 0.0405$, $R^2 = 0.9998$). A 0.5-ml aliquot of dilute extract (all the extracts were diluted with distilled water to adjust the absorbance within the calibration limits), 10 ml of distilled water, 1 ml of Folin–Ciocalteu reagent and 3 ml of Na_2CO_3 (20% w/v) were mixed in this order, made to 25 ml by distilled water and heated at 50 °C for 5 min. After 30 min, the absorbance was measured at 765 nm against a blank similarly prepared, but containing distilled water instead of extract. Similar calculations as in the previous section enable to express the results as the equivalent to milligrams of gallic acid per gram of skin (mg GAE/g).

2.3.4. Determination of total flavanols

The amount of total flavanols was determined after reaction with dimethylaminocinnamaldehyde (DMACA) in strong acid medium (Treutter, 1989; De Pascual-Teresa et al., 1998). The reagent was prepared by dissolving 100 mg of DMACA in 100 ml of methanol, 10% (v/v) in HCl. Two milliliters of this solution was added to 0.8 ml of extract and, after 10 min, the absorbance at 640 nm was measured against a blank prepared with 0.8 ml of extract and 2 ml of 10% (v/v) HCl in methanol.

(+)-Catechin was used as standard. A calibration curve was run with solutions of 5, 10, 15, 20 and 25 mg/l of this compound ($y = 0.1142x - 0.0073$, $R^2 = 0.9998$). All the extracts were diluted before reaction with DMACA to adjust the absorbance within the calibration limits. The results were expressed as milligrams of (+)-catechin equivalent per gram of skin (mg CE/g).

2.3.5. HPLC-DAD and HPLC-MS-ESI analysis

The individual separation of the analytes in both the extracts and standard solutions was performed on an Ultrabase C-18 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), using an injection volume of 20 μl and flow-rate of 1.7 ml/min. A binary gradient from a mobile phase A consisting of 10% (v/v) formic acid aqueous solution and a mobile phase B consisting of 10% (v/v) formic acid in acetonitrile was used with the following elution program: linear gradient from 10% to 27% B in 15 min and from 27% to 100% B in 30 min. The analytes were identified by comparing their retention times and UV spectra with those of the corresponding standards and quantified at the following wavelengths: 530 nm for anthocyanins, 320 nm for hydroxycinnamic acids and trans-resveratrol and 360 for quercetin.

HPLC-MS-ESI analysis was performed on a Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm i.d., 5 μm particle, Agilent, Barcelona, Spain) held at 40 °C, using an injection volume of 50 μl and flow-rate of 0.63 ml/min. A binary gradient from a mobile phase A consisting of water–acetonitrile–formic acid (83:7:10) and a mobile phase B consisting of water-acetonitrile-formic acid (40:50:10) was used with the following elution program: linear gradient from 6% to 20% B in 20 min, from 20% to 40% B in 15 min, from 40% to 60% B in 5 min, from 60% to 90% B in 5 min and isocratic mode at 90% B for 5 min. MS-ESI was used in positive mode with a capillary voltage of 2.5 kV, a cone voltage of 70 V, a vaporizer temperature of 350 °C, and a carrier gas flow (nitrogen) of 11 ml/min. The scan range was 50–1200 m/z.

3. Results and discussion

3.1. Study of the variables influencing extraction

A screening of seven variables was carried out in order to know their influence on extraction. Percentage of ethanol and HCl, temperature, extraction time, flow-rate, amount of sample and pressure were studied by a Plackett-Burmann design within the ranges shown in Table 1.

Table 1
Tested ranges of variables in each design and conditions selected

Variable	First design	Second design	Condition selected
Ethanol (%)	60–100	40–60	50
HCl (%)	0.2–0.8	0.8	0.8
Temperature (°C)	60–90	90–120	120
Time (min)	20–40	40–60	30
Flow-rate (ml/min)	0.8–1.2	1.2	1.2
Amount of sample (g)	1–3	1	1
Pressure (bar)	40–80	80	80

The data from total anthocyans and phenolics are in Fig. 2 as Pareto charts. Only two variables had significant effects for the former family: time (positive influence) and percentage of ethanol (negative influence). The trend was similar for total phenolics, but the effect of temperature and percentage of HCl were also significant in this case, both with positive influence. From these results, the three variables without significant influence were fixed following the trends in the Pareto charts. It was decided to select 0.8% HCl, despite its significant effect on the total phenolics extracted, because percentages above 1% HCl can lead to artifacts, as a result of hydrolysis of acylated anthocyanins during extraction (Rivas-Gonzalo, 2003).

A second multivariate approach was required; a complete factorial design of three variables in this case, in which the time interval and temperature range were increased and the ethanol percentage was decreased (Table 1). The analysis of the results for total anthocyans and phenolics showed that none of the three variables was significant. In both cases, the temperature and time had positive effects, but the influence of the ethanol percentage was negative for total anthocyans and positive for total phenolics. On the basis of these data, 120 °C was selected and kinetic extractions were carried out at this temperature with 40%, 50% and 60% of ethanol.

Fig. 3 shows that anthocyans were quantitatively extracted in 20 min, without significant differences when ethanol in the extractant is between 40% and 60%, but at least 30 min and 50% of ethanol were necessary for practically total extraction of phenolics; therefore, 30 min and 50% of ethanol were selected. Due to the obvious interest, from the economic and environmental points of view, of avoiding the use of organic solvents, a kinetics extraction study was also carried out under the previously optimized conditions, but using as extractant 0.8% (v/v) HCl in distilled water. The results in Fig. 3 indicate that ethanol accelerates drastically the extraction of anthocyans and phenolics, probably because it denatures cellular membranes and facilitates solubilization of these compounds (Jackman et al., 1987). Despite superheated acidic water extraction is clearly slower than SEWE, an in-depth study of the extraction time–ethanol costs would be necessary for

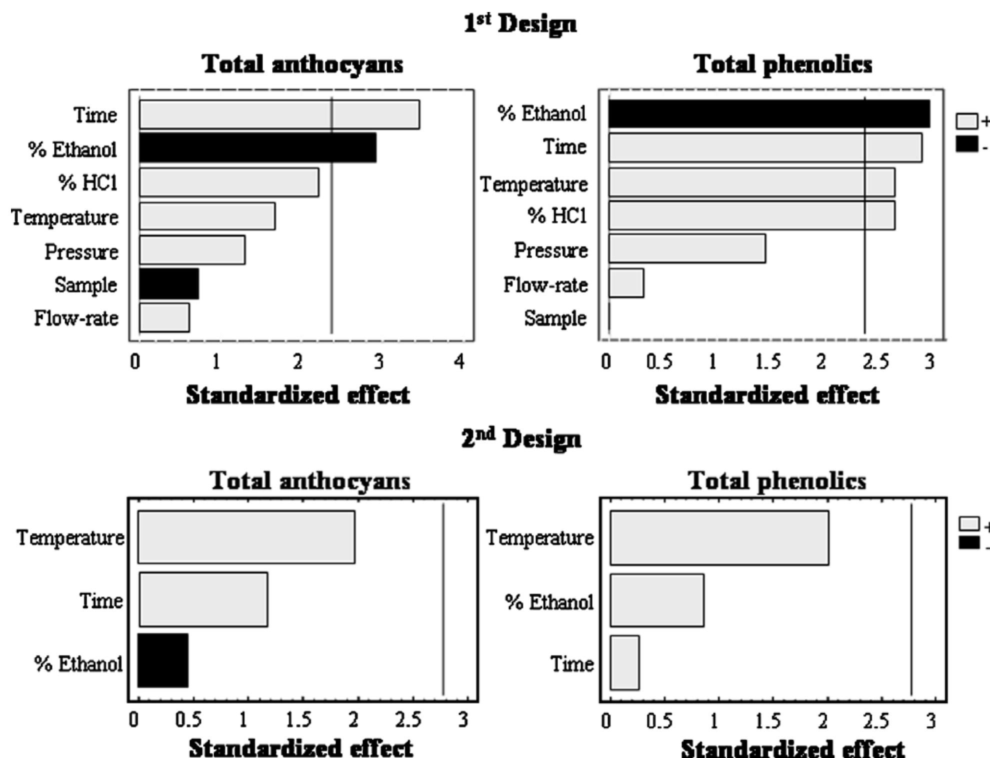

Fig. 2. Standardized Pareto charts for total anthocyans and phenolics.

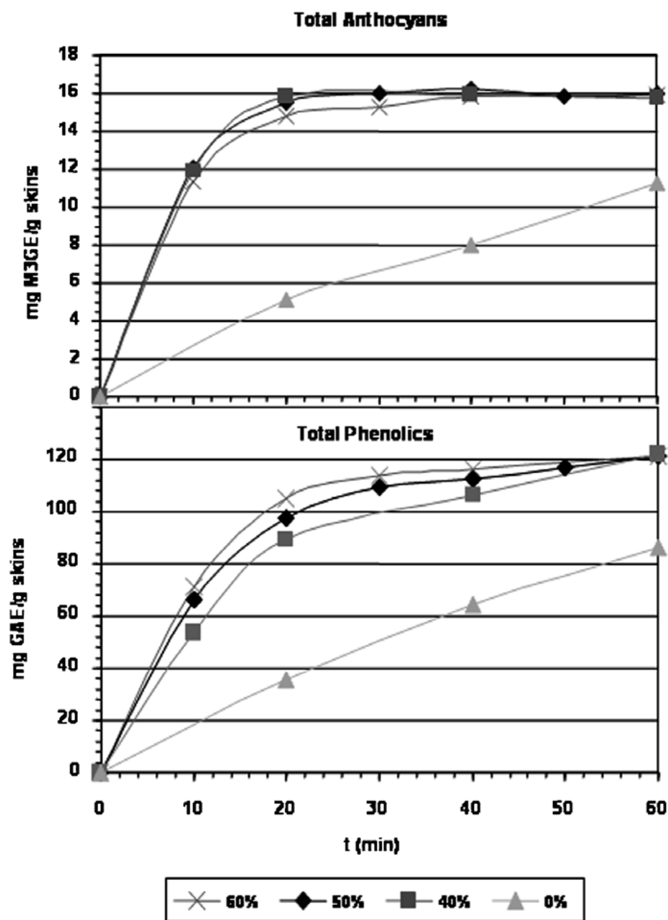

Fig. 3. Total anthocyans and phenolics extracted by SEWE with different percentages of ethanol (120 °C, 0.8% HCl, 1.2 ml/min, 80 bar).

potential implementation of the method at the industrial scale.

3.2. Precision of SEWE

The precision of the SEWE, expressed as repeatability and reproducibility, was calculated for total anthocyanins and phenolics. Within-day (intra-day) and between days (inter-day) assays were developed over a 7-day period. Two extractions under the selected working conditions were carried out every day, one in the early morning and other in the evening. The intra-day assay variability (RDS) was 5.9% for both anthocyans and phenolics and 6.5% for flavanols. The inter-day assay variability was 9.0% for anthocyans, 7.4% for total phenolics and 6.1% for flavanols.

3.3. Preparation of grape skins

Grape skins separated from grape pomace have a high level of moisture, which involves two shortcomings which restrict the extraction efficiency as compared with a dry material; (a) the yield for a given weight of sample is quite lower; consequently, the extracts obtained are less concentrated and the extractant consumption is higher for extracting the same amount of phenolic compounds; (b) the moisture hinders to grind skins to powder, also to the detriment of extraction efficiency.

On the other hand, the degradation rate of anthocyanins is time- and temperature-dependent so drying by heating at

Table 2
Influence of drying and grinding of grape skins on SEWE under the selected conditions

	Dried at 40 °C for 24 h		No dried	
	Ground	No ground	Ground	No ground
Anthocyans[a]	17	11	7	5
Total phenolics[b]	126	90	60	45
Flavanols[c]	35	23	16	11

[a] Data expressed as mg M3GE/g skin.
[b] Data expressed as mg GAE/g skin.
[c] Data expressed as mg CE/g skin.

temperatures higher than 70 °C must be avoided. For this reason, the most extended method for preparing grape skins to extract anthocyanins at a laboratory scale is freeze-drying. However, the cost of this method is expensive from the industrial point of view. An alternative to both methods may be to heat the grape skins at 40 °C, which would minimize degradation, partially eliminate the moisture and facilitate grinding. A 24-h drying-time was selected as compromise between time spent for this step and sample conditions for grinding.

With the aim of checking the feasibility of this alternative and evaluating the improvement of the efficiency regarding the use of skins without treatment, extractions were carried out under the conditions selected with 1-g skins samples prepared as follows: (a) dried at 40 °C for 24 h and ground; (b) dried at 40 °C without ground; (c) ground without drying; and (d) without ground nor drying. The results in Table 2 show clearly that soft heating increase enormously the yield of all compounds (increases of 140% for anthocyanins, 100% for total phenolics and 118% for flavanols as compared with samples without grinding; 143% for anthocyanins, 110% for total phenolics, 119% for flavanols as compared with ground samples). Concerning the effect of grinding, the yield was higher for dried skins as compared with no dried skins (54% vs. 40% for anthocyanins, 40% vs. 33% for total phenolics and 52% vs. 45% flavanols), probably due to the smaller particle size in the first case.

3.4. Yield and composition of SEWE extracts

SEWE extracts were analysed by HPLC with both UV–visible and MS detectors with the results shown in Table 3. The first outstanding aspect is that the total amount of anthocyans obtained by HPLC was around 30% of that obtained by direct spectrophotometric measurement. Thus, when identified and no identified peaks of the SEWE chromatogram were quantified using calibration with Mv3G, the result was 5967 ± 228 μg/g; however, $17{,}510 \pm 1571$ μg/g was obtained by spectrophotometry. This fact involves that 2/3 of anthocyans were not free anthocyanins, but they are linked to other molecules forming higher molecular-weight anthocyanin-derivatives, which are retained by the filter before the HPLC analysis. This predominance of polymeric pigments is a positive aspect of SEWE extracts by SEWE as they are more stable than monomeric anthocyanins – viz. anthocyanins-derivative pigments are responsible for maintaining colour intensity and adding violet hues in aged wines (Pérez-Magarino and González-San José, 2004).

Other outstanding aspect is the anthocyanins percentage. Of the total amount of monomeric anthocyans, 53.8% were *p*-coumaroyl derivatives (42.2% Cm-Mv3G), 20.9% 3-glycosides and 9.9% aglycons. The predominance of *p*-coumaroyl derivatives in the SEWE extract was pre-

Table 3
Yield of SEWE and comparison with dynamic conventional solid–liquid extraction

	SEWE	DNCE[a]
Spectrophotometry		
Total anthocyans[b]	$17{,}510 \pm 1571$	5755
Total phenolics[c]	126 ± 9	18
Total flavanols[d]	35 ± 1	3
HPLC-UV		
Total anthocyans[b]	5967 ± 228	4424
Dp3G[e]	93.2 ± 3.8	43.4
Cy3G[e]	16.0 ± 0.8	2.0
Pt3G[b]	136.2 ± 4.6	79.2
Pn3G[e]	45.4 ± 1.7	11.2
Mv3G[e]	957.1 ± 32.4	635.4
Dp[e]	70.5 ± 1.9	–
Cy[e]	439.5 ± 25.2	12.7
Pt[e]	17.7 ± 2.4	–
Pn[e]	8.6 ± 1.4	–
Mv[e]	53.3 ± 5.4	–
Cf-Mv3G[b]	122.2 ± 8.3	101.6
Cm-Dp3G[f]	352.7 ± 23.4	337.1
Cm-Pt3G[b]	218.7 ± 10.7	346.2
Cm-Pn3G[f]	122.3 ± 3.3	109.6
Cm-Mv3G[b]	2519.1 ± 116.1	2571.7
4-VC-Mv3G (Pinotin A)[b]	19.6 ± 0.8	13.8
4-VC-Cm-Mv3G[b]	7.4 ± 0.7	3.7
Flavonols		
My[g]	121.1 ± 7.1	4.4
Qr[g]	236.1 ± 28.7	123.4
n.i.[g]	18.2 ± 1.9	8.6
Kp[g]	94.1 ± 6.5	46.8
Is[g]	32.5 ± 4.5	18.0
MyG[g]	23.7 ± 2.1	15.0
QrGluc[g]	86.7 ± 4.1	76.1
QrG[g]	12.4 ± 1.4	11.5
Other compounds		
Caffeic acid[e]	14.9 ± 0.9	12.5
p-Coumaric acid[e]	21.2 ± 2.6	12.0
Resveratrol[e]	9.6 ± 0.9	4.1

[a] Dynamic normal conditions solid–liquid extraction.
[b] Data expressed as μg M3GE/g skin.
[c] Data expressed as mg GAE/g skin.
[d] Data expressed as mg CE/g skin.
[e] Data obtained with the corresponding standard expressed as μg compound/g skin.
[f] Data obtained with the standard of the corresponding glycoside and expressed as μg glycoside/g skin.
[g] Data expressed as μg QrE/g skin. n.i. no identified.

dictable, since they are the less polar anthocyanins and so the most difficult to be extracted during winemaking. On the other hand, the higher polarity of 3-glycosides explains their lower proportion in the SEWE extract. As for anthocyanin aglycons, their presence is a consequence of temperature, as discussed in the following section. Two pyranoanthocyans (4-vinylcatechol-Mv3G, 4-VC-Mv3G, and 4-vinylcatechol-Cm-Mv3G, 4-VC-Cm-Mv3G) were also detected; compounds which are absent in fresh grapes, result from the reaction of Mv3G and Cm-Mv3G with free caffeic acid and are detected in 2.5- to 4-year-old wines (Schwarz et al., 2004, 2005). Finally, the absence of acetylated anthocyans was probably due to acid hydrolysis by HCl.

Concerning flavonols, myricetin (My), quercetin (Qr), kaempferol (Kp), isorhamnetin (Is), myricetin-3-*O*-glycoside (MyG), quercetin-3-*O*-glycoside (QrG), quercetin-3-*O*-glucuronide (QrGluc) and a non-identified flavonol aglycon, which is detected in some very aged wines, were detected and quantified. Qr and its derivatives are the most abundant flavonols, with a high percentage (80%) of free aglycons, which testifies that hydrolysis takes place during extraction as grapes only contain the glycosilated forms of flavonols.

3.5. Comparison of SEWE and dynamic normal-working conditions solid–liquid extraction (DNCE)

Extraction carried out in the same system and under the same working conditions as SEWE but a room temperature and atmospheric pressure showed that superheated conditions increase the hydrolysis of anthocyanins and flavonols glycosides. This fact is clear for anthocyanins from the presence of free anthocyanins aglycons in SEWE extracts, which were absent in the extract from DNCE (Table 3). As for flavonols, glycoside–aglycon ratios were higher for DNCE than for SEWE; however, this trend is compensated by the highest efficiency of SEWE for all compounds but Cm-Pt3G, which was lower, and Cm-Dp3G and Cm-Mv3G, with not significant differences. Particularly high (300% over the DCNE) was the increase of total anthocyans extracted by SEWE, corresponding 165% to anthocyanin-derivative pigments and 135% to monomeric anthocyans; therefore, DNCE predominantly extracts monomeric anthocyans (76.9% vs. 34.1% of SEWE). Finally, total phenolics and flavanols yields were enormously higher (7.0- and 11.7 times, respectively) for SEWE.

3.6. Comparison between SEWE and commercial extracts from red grape skins

The composition of SEWE extracts was compared with those of two commercial red-grape skin extracts from Australia, named as GST and GSTA. The chromatograms in Fig. 4 shown the anthocianins profiles of the three extracts and Table 4 total anthocyans, phenolics and flavanols as obtained by direct spectrometry, as well as the concentrations of all compounds identified by HPLC. No data are available about the amount, type and characteristics of the skins used to obtain the commercial extracts.

The spectrophotometric data demonstrated that the proportion of total flavanols with respect to total anthocyans and phenolics is much higher in SEWE extracts. The concentration of total anthocyans and total phenolics was approximately 10 times higher in both commercial extracts, while that of total flavanols was only 2.4 times higher. The percentage of monomeric anthocyanins in total anthocyans was also higher in SEWE extracts (34.4% in SEWE vs. 17.4% and 20.6% in GSTA and GST, respectively).

The practically total absence of acylated anthocyanins and pyranoanthocyans and the greater (3 times) proportion of free anthocyanins aglycons in the commercial extracts were two substantial differences with respect to SEWE extracts. Acylated anthocyanins were more than half of the monomeric anthocyanins and free anthocyanins aglycons were only 10.0%, 3 times less than in the GST and GSTA extracts. These are positive aspects of SEWE extracts as acylation increases the stability of

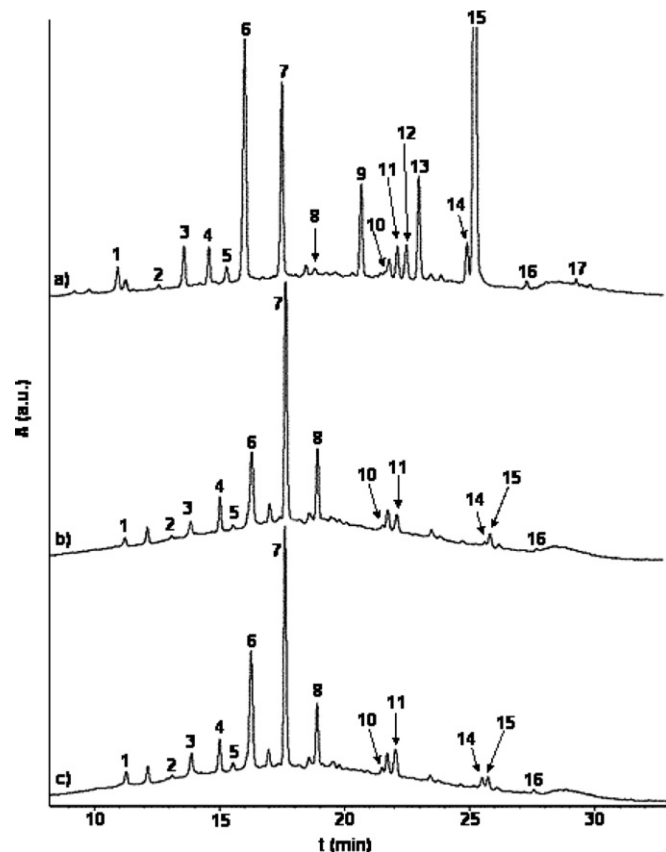

Fig. 4. Chromatograms at 530 nm of (a) SEWE; (b) GSTA (5 times diluted); (c) GST (5 times diluted). Compounds: **1**, Dp3G; **2**, Cy3G; **3**, Pt3G; **4**, Dp; **5**, Pn3G; **6**, Mv3G; **7**, Cy; **8**, Pn; **9**, Cm-Dp3G; **10**, Pt; **11**, Mv; **12**, Cf-Mv3G; **13**, Cm-Pt3G; **14**, Cm-Pn3G; **15**, Cm-Mv3G; **16**, 4-VC-Mv3G; **17**, 4-VC-Cm-Mv3G.

Table 4
Comparison of commercial and SEWE extracts

	SEWE	GSTA	GST
Spectrophotometry			
Total anthocyans[a]	432.6	3872.5	4504.7
Total phenolics[b]	3091.8	27104.0	31868.0
Total flavanols[c]	803.5	1853.3	2032.4
HPLC-UV			
Total anthocyans[a]	148.9	673.6	929.5
Dp3G[d]	2.3 (1.6)	7.4 (1.1)	20.9 (2.3)
Cy3G[d]	0.4 (0.3)	3.9 (0.6)	6.8 (0.7)
Pt3G[a]	3.4 (2.3)	14.3 (2.1)	30.9 (3.3)
Pn3G[d]	1.1 (0.8)	3.0 (0.4)	9.0 (1.0)
Mv3G[a]	23.9 (16.0)	96.3 (14.3)	180.9 (19.5)
Total 3-glycosides	31.1 (20.9)	125.0 (18.6)	248.5 (26.7)
Dp[d]	1.8 (1.2)	17.8 (2.6)	22.6 (2.4)
Cy[d]	11.1 (7.4)	159.0 (23.6)	178.8 (19.2)
Pt[d]	0.4 (0.3)	42.1 (6.3)	49.6 (5.3)
Pn[d]	0.2 (0.1)	0.3 (0.1)	3.1 (0.3)
Mv[d]	1.3 (0.9)	10.2 (1.5)	22.2 (2.4)
Total aglycones	14.8 (10.0)	229.4 (34.1)	276.3 (29.7)
Cf-Mv3G[a]	3.1 (2.0)	–	–
Cm-Dp3G[e]	8.8 (5.9)	–	–
Cm-Pt3G[a]	5.5 (3.7)	–	–
Cm-Pn3G[e]	3.0 (2.0)	–	0.2 (0.02)
Cm-Mv3G[a]	62.8 (42.2)	–	1.4 (0.2)
Total *p*-coumaroyl derivatives	80.1 (53.8)	–	1.6 (0.2)
4-VC-Mv3G[a] (Pinotin A)	0.5 (0.3)	–	0.3 (0.03)
4-VC-Cm-Mv3G[a]	0.2 (0.1)	–	–
Total pyranoanthocyans	0.7 (0.5)	–	0.3 (0.03)
Flavonols			
My[f]	3.0	6.0	2.0
Qr[f]	5.9	10.8	5.9
n.i.[f]	0.5	0.5	0.1
Kp[f]	2.4	1.8	0.8
Is[f]	0.7	2.3	1.1
MyG[f]	0.6	7.3	4.4
QrGluc[f]	2.2	21.9	9.8
QrG[f]	0.3	8.9	3.2
Other compounds			
Caffeic acid[d]	0.4	6.5	2.2
p-Coumaric acid[d]	0.5	3.2	2.0
Resveratrol[d]	0.2	0.7	1.0

[a] Data expressed as mg M3GE/l.
[b] Data expressed as mg GAE/l.
[c] Data expressed as mg CE/l.
[d] Data obtained with the corresponding standard expressed as mg compound/l.
[e] Data obtained with the standard of the corresponding glycoside and expressed as mg glycoside/l.
[f] Data expressed as mg QrE/l. n.i. no identified. Percentage of each anthocyanin with respect to the total monomeric anthocyanins content in brackets.

anthocyanins (Bassa and Francis, 1987) and results in more intense colour (Giusti et al., 1999). By contrast, percentages of monoglycosides were similar in the three extracts.

As for flavanols, SEWE extracts had similar or higher concentrations of free aglycons, except for isorhamnetin. Concentrations of identified glycosides were much higher in commercial extracts, particularly in GSTA, as well as those of hydroxycinnamic acids and resveratrol.

4. Conclusions

This research demonstrated the feasibility of using superheated ethanol–water mixtures to obtain extracts from winemaking residues (grape skins) which are rich-in-anthocyanins and other polyphenols and can be used as natural colorants or for nutraceutical purposes. Skins from grape pomace were extracted, unlike previous works on this subject where skins of fresh grapes were the samples; therefore, the results obtained are closer to the real situation for exploitation of these residues. Under the selected working conditions, 30 min were enough for total extraction of these pigments and for obtaining extracts with a qualitative composition of anthocyanins better (from the point of view of stability and colour intensity) than two commercial grape skin extracts. Also, the use of superheated acidified water as extractant was tested but the efficiency of the extraction was much lower. Consequently, the extraction method here reported, feasible at a laboratory-scale, could also be a fast alternative to the conventional industrial extraction of high added-value compounds from grape pomace.

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