Determination of Anthocyanins in Red Grape Skin by Pressurised Fluid Extraction and HPLC

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Abstract: Grape anthocyanins not only play an important role in the colour quality of red wines but they also have many beneficial effects on human health, e.g., reduction of coronary heart disease incidence, or anticarcinogenic and antioxidant properties. Therefore, a rapid and efficient extraction technique prior to chromatographic analysis is of primary interest. Pressurised Fluid Extraction (PFE) presents a fast, effective, and environmentally friendly extraction method for the analysis of red grape pigments. In this study, PFE in static mode was utilised for the extraction of 3-monoglucoside anthocyanins from the grape skin of highly pigmented variety Alibernet. The effects of the type of the extraction solvent and the extraction temperature were studied. The identification of the above given compounds were performed by high-performance liquid chromatography with diode array detection (HPLC-DAD) based on Synergi C-12 column separation. The wavelength was set at 520 nm. All compounds were determined and identified during 50 minutes.

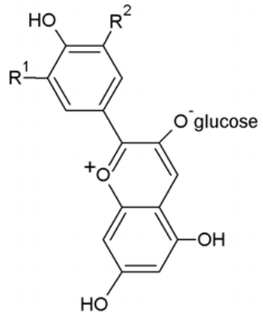
Keywords: flavonoids; grapes; PFE; HPLC analysis

Anthocyanins are a group of naturally occurring phenolic compounds that are responsible for the colouration of fruits, vegetables, and flowers. They have also many beneficial effects for humans including the reduction in the incidence of coronary heart disease, enhancement of visual acuity, maintenance of normal vascular activity, as well as anticarcinogenic, antimutagenic, anti-inflammatory, and antioxidative properties. Therefore, the determination of anthocyanins in red grapes and wines has acquired of increasing interest during last years. Grape anthocyanins play an important role in the colour quality of red wine and they are also successfully used as food colourants and nutraceuticals (Espín *et al.* 2007).

Grape anthocyanins are traditionally extracted with methanol, ethanol, acetone, or their aqueous mixtures. In most cases, the solvents are acidified with HCl or various organic acids, e.g. formic, acetic, or trifluoroacetic acids in different concentrations. Acidic solvents denature cellular membranes and facilitate the solubilisation of anthocyanins (Revilla *et al.* 1998; Naczk & Shahidi 2004).

Since the extraction is the first step in the commercial isolation of anthocyanins, a rapid and efficient extraction technique prior to chromatographic analysis is nowadays of primary interest. The extraction at elevated temperature and pressure, Pressurised Fluid Extraction (PFE) represents an ideal technique for a rapid extraction of grape anthocyanins. The elevated pressure keeps the solvents in the liquid state above their boiling points and allows the high-temperature extraction. The higher temperature increases the diffusion rate, solubility, and mass transfer of the compounds, improves the contact of the analytes with the solvents, and therefore the extraction can be achieved more rapidly with lower solvent consumption as compared with the conventional extraction methods (Richter *et al.* 1996). Ju and

Supported by the Czech Science Foundation (Grant No. GA 203/08/1536) and by the Academy of Sciences of the Czech Republic (Institutional Research Plan No. AV 0Z40310501).



Compound	R^1	R^2
Dpd3glc	OH	OH
Cyd3glc	OCH_3	OH
Ptd3glc	OCH_3	H
Pnd3glc	OCH_3	OCH_3
Mvd3glc	OH	H

Figure 1. Structures of 3-monoglucosides anthocyanins

Howard (2003) investigated the effects of different solvents and temperatures on PFE of anthocyanins from the skin of highly pigmented red wine grapes. They concluded that 60% acidified methanol at 60°C extracted the highest levels of anthocyanins.

In this study, we investigated how much different solvents and temperatures affect the extraction of 3-monoglucoside anthocyanins malvidin, delphidin, peonidin, cyanidin, and petunidin (Figure 1) from freeze-dried grape skin of the highly pigmented variety Alibernet. The identification of the above given compounds were performed using high-performance liquid chromatography with diode array detection (HPLC-DAD) based on a C-12 column separation.

MATERIALS AND METHODS

Chemicals and standards. Acetonitrile, methanol, ethanol, water, and formic acid, all HPLC-grade, were purchased from Sigma-Aldrich (Prague, Czech Republic). Plastic bag was also supplied by Sigma-Aldrich (Prague, Czech Republic). Dry ice, liquid nitrogen and plastic bowes were purchased from Linde Gas, a.s. (Brno, Czech Republic).

A mixture of anthocyanins standards containing 3-monoglucosides of delphidine (Dpd3glc), cyanidin (Cyd3glc), petunidin (Ptd3glc), pelargonidin (Pgd3glc), peonidin (Pnd3glc) and malvidin (Mvd3glc) was purchased from Polyphenols Laboratories AS (Sandnes, Norway).

Sample preparation. Grapes of the variety Alibernet were collected in a vineyard located in Mikulov (region south Moravia, Czech Republic). The harvested grapes were placed into plastic bowes containing dry ice and were transported to the laboratory. The grape skin was then manually separated under inert atmosphere and freeze-dried. The dried skin was ground to a powder under liquid nitrogen, placed in brown glass vials and stored at –20°C.

PFE of anthocyanins. The dry grape skin was extracted using PFE in a static mode employing *one*PSE extractor (Applied Separations, Allentown, USA). 0.5 g grape skin portions were placed into 11 ml extraction cells containing glass beads (570–700 µm) at the bottom of each cell. The PFE parameters were set as follows: temperature 40–120°C, pressure 15 MPa, extraction time 3 × 5 min, rinsing time 20 s, and nitrogen purge time 120 s after each extraction run. The solvents methanol and ethanol were tested for their ability to extract the anthocyanins from the grape skin. After the PFE run, the extracts were cooled to 5°C and stored in a fridge until HPLC analysis.

Soxhlet extraction. For comparison with PFE, the extraction of anthocyanins was also performed in Kavalier extraction apparatus (Sklárny Kavalier, a.s., Czech Republic). The grape skin sample (0.5 g) was placed in a 100 x 16 mm i.d. cellulose extraction thimble and extracted using 100 ml of methanol or ethanol for 1.5 hours. After the extraction, the extracts were concentrated to 35 ml volume, cooled to 5°C, and stored in the fridge until HPLC analysis.

HPLC analysis. HPLC apparatus was equipped with the gradient pump P 4000, autosampler AS 3000, and spectrophotometer detector UV 6000 LP (Spectra SYSTEM, USA). The wavelength was set at 520 nm. The separation of anthocyanins was performed on 4.6 mm i.d., Synergi C12 Max-RP 250 mm long column, 4 µm stationary phase (Phenomenex, USA). The mobile phase was a linear gradient of water:acetonitrile mixture 97:3 (solvent A) and 40:60 (solvent B) adjusted to pH 1.8 by formic acid, at a flow rate of 0.5 ml/min: 0 min 6% B; 20 min 20% B; 35 min 40% B; 40 min 60% B; 45 min 90% B; 47–55 min 6% B. LC systems were connected to PC and controlled by Chromquest software (Spectra SYSTEM, USA). All extracts were filtered through 0.45 µm syringe filter prior to LC analysis.

The anthocyanins were identified by comparison to their retention times with those of the standards. The monoglucosides anthocyanins were quantified using authentic standards of Dpd3glc, Cyd3glc, Ptd3glc, Pgd3glc, Pnd3glc, and Mvd3glc.

RESULTS AND DISCUSSION

Anthocyanins were extracted from the grape skin of the highly pigmented variety Alibernet by PFE at different temperatures (40–120°C) using methanol and ethanol, both in their pure state, as extraction solvents. PFE technique was compared with conventional Soxhlet extraction. The extracts were analysed by HPLC-DAD employing C12 columns. Five 3-monoglucosides anthocyanins, Dpd, Cyd, Ptd, Pnd, and Mvd, were identified by comparison of their retention times with those of the standards (Figure 2). The detection limit was for malvidin-3-glucoside and delphidin-3-glucoside 488 ng/l, and 244 ng/l for peonidin-3-glucoside, cyanidin-3-glucoside and petunidin-3-glucoside.

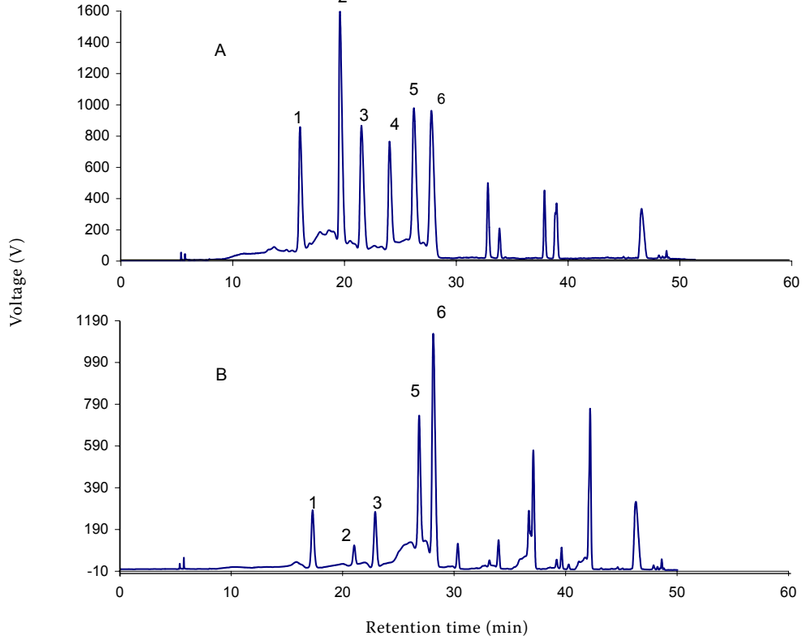


Figure 2. HPLC chromatograms of anthocyanins. (A) standard of 3-monoglucosides anthocyanins, (B) PFE extract from grape skin at 40°C with methanol
For experimental conditions see Materials and Methods. Peaks: (1) delphidin-3-glucoside, (2) cyanidin-3-glucoside, (3) Petunidin-3-glucoside, (4) pelargonidin-3-glucoside, (5) peonidin-3-glucoside, (6) malvidin-3-glucoside

The results obtained are shown in Table 1. When methanol was used as the solvent, maximum extraction of 3-monoglucosides anthocyanins occurred at 40°C. With increasing temperature, a decrease of the anthocyanins content was observed. If ethanol was used as the extraction solvent, the highest recovery was observed at 80°C. At higher temperatures, antocyanins degradation was observed, however, the extract obtained at 120°C had a higher anthocyanins content than that obtained at 40°C. In contrast to the results obtained with methanol, the anthocyanins recovery achieved with ethanol was two or three times lower for almost all monoglucosides anthocyanins.

The content of anthocyanins in the extracts obtained by Soxhlet extraction was two times lower than that in the extracts obtained by PFE at 60°C with methanol and 80°C with ethanol.

Compared to other wine grapes and extraction techniques, the PFE Alibernet extract gained by using two solvents was rich in monoglucoside anthocyanins, e.g. malvidine-3-glucoside content ranged from

Table 1. Amount of 3-monoglucosides anthocyanins (mg/g) in 1 g Alibernet grape skin obtained by PFE in comparison with Soxhlet extraction

Extraction	Solvent	Temperature (°C)	Dpd3glc	Cyd3glc	Ptd3glc	Pnd3glc	Mvd3glc
PFE	methanol	40	90.5	11.9	73.3	145	244
		60	33.3	2.28	24.9	53.9	98.4
		80	26.2	0.89	20.6	44.4	87.6
		100	31.6	1.83	24.1	48.3	90.9
		120	25.3	0.73	20.4	45.5	84.9
	ethanol	40	9.11	0.44	10.4	23.0	46.4
		60	13.2	0.71	12.4	27.2	60.1
		80	15.4	0.78	13.9	28.3	63.3
		100	11.9	0.48	10.8	19.3	48.6
		120	10.2	0.52	11.3	20.0	49.1
Soxhlet	methanol	64.7	13.0	0.50	12.1	20.5	43.4
	ethanol	78.3	6.91	0.38	7.54	19.7	31.3

Dpd3glc = delphidin-3-glucoside, Cyd3glc = cyanidine-3-glucoside, Ptd3glc = petunidin-3-glucoside, Pnd3glc = peonidine-3-glucoside, Mvd3glc = malvidin-3-glucoside

46 mg/g to 244 mg/g which is much higher than the literature values of the varieties Cabernet Sauvignon (0.38–0.78 mg/g), Arkansas (12–21 mg/g), and Merlot (0.485–1.137 mg/g) grape skins extracted by different extraction procedures with acidified or neutral solvents, PFE using acidified 60% methanol, and maceration with acidified 50% methanol, respectively (Revilla *et al.* 1998; Mazza *et al.* 1999; Ju & Howard 2003). These results indicate that the variety Alibernet grape skin can be used for anthocyanins and colour enhancement of food.

CONCLUSION

PFE in static mode was utilised for the extraction of monoglucoside anthocyanins from the Alibernet grape skin. It was shown that the solvent type, higher temperature, and higher pressure significantly affect the recovery of anthocyanins. Moreover, the time required for the extraction of anthocyanins from the grape skin was only 15 minutes. PFE could be a very efficient and powerful tool in the food industry for the extraction of grape anthocyanins.

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