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Research Note

Rapid isolation of malvidin 3-glucoside from red wine by high speed countercurrent chromatography (HSCCC)

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Summary: Rapid isolation of malvidin 3-glucoside from red wine was achieved by High Speed Countercurrent Chromatography (HSCCC). Red wine pigments were cleaned-up on an Amberlite XAD-7 column prior to separation by HSCCC. Separation of the anthocyanin isolate yielded 65 mg of pure malvidin 3-glucoside per liter red wine in a single run. Purity and identity of the isolated compound was checked by HPLC-DAD, ESI-MS/MS and ^{1}H-NMR. The method can be applied to red wine as well as grape skin extracts.

Key words: red wine, anthocyanins, malvidin 3-glucoside, countercurrent chromatography.

Introduction: Countercurrent chromatography (CCC) is an all-liquid chromatographic technique that has been applied to the separation of many natural compounds (Ito and Petroski 1995). A detailed description of the principle of CCC is presented by Ito (1986), Mandava and Ito (1988) and Conway (1990). CCC offers various advantages compared to conventional liquid chromatographic techniques. These are the gentle operation conditions, high sample loads, good separation power and the absence of a solid stationary phase, which minimizes the risk of artifact formation. Separation of anthocyanins from various foodstuff by HSCCC has recently been described (Degenhardt *et al.* 2000). Since pure reference compounds were required for quantification of anthocyanins and commercial standards were only available at a high price, a rapid method for the preparative isolation of malvidin 3-glucoside was developed. Details are described here.

Material and Methods: A Californian red wine "Western Cellars" (vintage 1998) and "Hex vom Dasenstein", a Spätburgunder (Pinot noir) red wine, Kappelrodeck, Baden (1997) were used. After evaporation of ethanol *in vacuo*, the dealco-holised wine was applied onto a column (50 cm x 4 cm) of Amberlite XAD-7 (Fluka Chemie, Buchs, Switzerland). The column was washed with 1 l of water to remove sugars and organic acids. The elution of the pigments was carried out with methanol:acetic acid (19:1, v:v). Methanol was evaporated *in vacuo*, and the aqueous solution partitioned twice against 300 ml of ethyl acetate to remove other phenolics. The aqueous phase was lyophilised to yield approximately 1 g of a red powder. The freeze-dried pigments were separated by HSCCC without further treatment.

A high speed Model CCC-1000 of Pharma-Tech Research Corporation (Baltimore, Maryland, USA) was equipped with 3 preparative coils, connected in series (total volume: 850 ml). The separations were run at 1000 rpm and at a flow rate of 5 ml·min^{-1}. A Biotronik HPLC pump BT 3020 was used to pump the liquids. All samples were dissolved in a 1:1 mixture of light and heavy phase and injected into the system by loop injection. Stationary phase retention was in the range of 53-80 %. The solvent system consisted of TBME:*n*-butanol:acetonitrile:water (2:2:1:5, acidified with 0.1 % TFA) with the less dense organic layer acting as stationary phase. Elution mode was head to tail (cf. Degenhardt *et al.* 2000).

HPLC with diode array detection (HPLC-DAD): Jasco ternary gradient unit LG-980-02, with degasser and MD-910 multiwavelength detector driven by BORWIN chromatography software has been used. Peak detection was carried out at 320 and 520 nm. Spectra were also visualised as contour plot in the wavelength region 220-550 nm. The chromatographic separation was performed on a LUNA RP18 5 µm column (150 mm x 4.6 mm; Phenomenex, Aschaffenburg, Germany) at ambient temperature. The mobile phase was a linear gradient of 10 % aqueous formic acid (solvent A) and acetonitrile:10 % aqueous formic acid (9:1, v:v; solvent B). Conditions: initial 95 % A, 5 % B; in 40 min to 50 % A, 50 % B; after flushing with 100 % B back to initial conditions; flow rate: 0.5 ml·min^{-1}.

Proton magnetic resonance spectroscopy (^{1}H-NMR): All experiments were performed on a Bruker AMX 300 spectrometer (300 MHz). Spectra were recorded in CD_3OD-CF_3COOD (19:1, v:v). Assignments were made on the basis of spectral data published by Pedersen *et al*. (1993).

Electrospray ionization ion trap multiple mass spectrometry (ESI-MS/MS): Bruker Esquire-LC-MS/MS with electrospray ionization in the positive mode was used. The sample was introduced as solution in acetonitrile:water (3:2; v:v) using a syringe pump. Dry gas was nitrogen with a gas flow of 4 l·min^{-1} (350 °C); the nebulizer was set at 10 psi. The parameters were: capillary: -2500 V, end plate: -2000 V, capillary exit: 110 V, skim 1: 35 V, skim 2: 8 V. MS/MS-experiments were performed with different fragmentation amplitudes.

Malvidin 3-glucoside: UV-VIS maxima (determined by HPLC-DAD): 279, 347, 527 nm; ESI-MS/MS: molecular ion at *m/z* 493 $[M]^+$, most prominent daughter ion of 493: *m/z* 331 $[M\text{-}Glc]^+$; ^{1}H-NMR (300 MHz, CD_3OD/TFA (d_1) 19:1, v:v, in ppm): δ 9.02 (1 H, s, H4); 7.97 (2 H, s, H2'/H6'); 6.94 (1 H, d, J = 1.5 Hz, H6); 6.66 (1 H, d, J = 1.5 Hz, H8); 5.34 (1 H, d, J = 8 Hz,

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H1“); 4.03 (6 H, s, OMe); sugar resonances from 3.94 to 3.40 ppm. Assignments were made according to PEDERSEN *et al.* (1993).

Results and Discussion: Analysis of the anthocyanin fraction of red wines has importance for the discrimination of grape varieties. In addition, it allows the detection of hybrid grapes as well as the adulteration of red wines (HOLBACH *et al*. 1997). For the analysis of anthocyanins by HPLC authentic reference compounds are required. Although some standards are commercially available, we were interested in a rapid method to isolate anthocyanins from red wine on a preparative scale.

The separation of malvidin 3-glucoside from other red wine pigments is shown in Figs.1 and 2. In the case of the Californian red wine 65 mg of malvidin 3-glucoside was obtained from one liter of red wine. Peak purity and identity control was made by ^{1}H-NMR, ESI-MS/MS and HPLC-DAD. According to the spectra, the isolated malvidin 3-glucoside is pure enough to serve as a standard

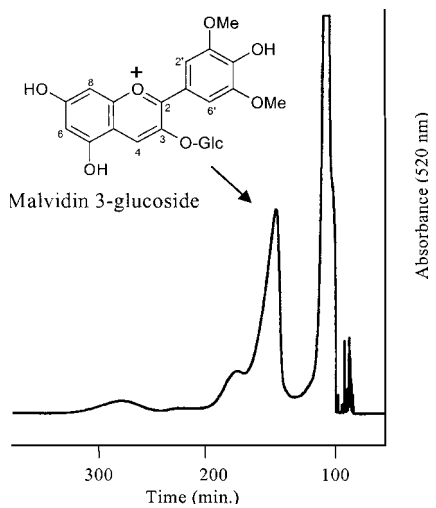

Fig.1: Preparative HSCCC separation of the XAD-7 enriched anthocyanin fraction of a Californian red wine.

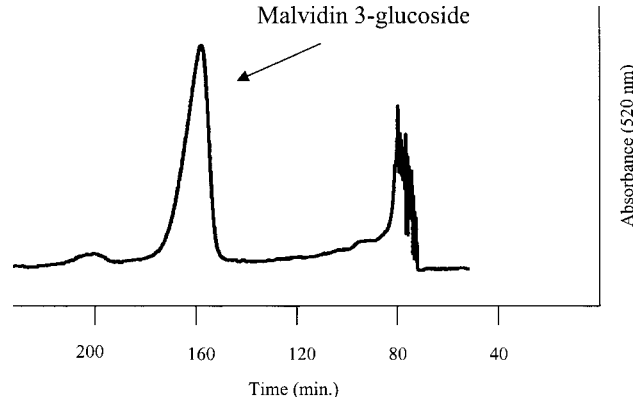

Fig. 2: Preparative HSCCC separation of the anthocyanin fraction of a Spätburgunder red wine.

reference compound. In contrast to the Californian red wine, the anthocyanin profile of the German Spätburgunder wine was much simpler with the major pigment also being malvidin 3-glucoside (MAZZA and MINIATI 1993). Isolation of other red wine pigments is currently under way and will be reported soon.

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