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Comparison of high-performance liquid chromatography separation of red wine anthocyanins on a mixed-mode ion-exchange reversed-phase and on a reversed-phase column

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ABSTRACT

Anthocyanins, which confer the characteristic color to red wine, can be used as markers to classify wines according to the grape variety. It is a complex separation that requires very high chromatographic efficiency, especially in the case of aged red wines, due to the formation of pyranoanthocyanins. A coelution between these kinds of compounds can affect the $R_{ac/coum}$ ratio of aged wines, and might lead to false results when classifying the wine variety. In 2007, the use of a novel mixed-mode ion-exchange reversed-phase column was reported to separate anthocyanins extracted from grapes of *Vitis labrusca* with different selectivity than C-18 columns. In the present work, the separation of anthocyanins including pyranoanthocyanins in young and aged Cabernet Sauvignon wines and other varieties is evaluated. The most interesting contributions of this research are the different elution order and selectivity obtained for anthocyanins and pyranoanthocyanins (only formed in wine), compared with those observed in C-18 stationary phases. Also interesting is the separation of the polymeric fraction, which elutes as a clearly separated peak at the chromatogram's end. However, a comparison with a high efficiency C-18 column with the same dimensions and particle size demonstrated that the tested mixed-mode column shows broader peaks with a theoretical plate number below 8000, for malvidin-3-glucoside peak, while it can be up to 10 times higher for a high efficiency C-18 column, depending on the column manufacturer. Under the tested conditions, in mixed-mode phase, the analysis time is almost twice that of a C-18 column with the same dimensions and particle size. A mixed-mode phase with increased efficiency should provide an interesting perspective for separation of anthocyanins in wine, due to its improved selectivity, combined with a useful role in a second-dimension separation in preparative anthocyanin chromatography.

1. Introduction

Anthocyanins confer the characteristic color to red wine [1]. These pigments are well known for the health benefits associated with the moderate consumption of red wine [2]. Anthocyanins belong to the flavonoid group of polyphenolic compounds. Naturally occurring anthocyanins are composed of six anthocyanidin aglycones linked to sugar groups at positions 3 and/or 5 [3]. Some of the chemical structures of the occurring anthocyanins and pyranoanthocyanins in wine are shown in Fig. 1.

Anthocyanins can be use as chemical markers for varietal differentiation in red wines, where a common method uses the ratio of acetylated and coumaroylated anthocyanins ($R_{ac/coum}$) according to Holbach et al. [4].

Determination of the grape variety of red wines by high-performance liquid chromatography (HPLC) requires accurate analytics, especially in the case of aged red wines. With the multiple chemical reactions that occur during wine maturation, the anthocyanin profile changes, due to the formation of pyranoanthocyanins [5,6], such as the reaction product of mv-3-gl and pyruvic acid, vitisin A and pinotin A, a product of mv-3-gl and caffeic acid [7–9]. These processes occur during wine elaboration and therefore there are no pyranthocyanins in grapes.

Under the commonly used HPLC conditions for anthocyanin analysis of red wine using C-18 columns [9–15], pinotin A coelutes with the coumaroylated derivatives of mv-3-gl and pe-3-glucoside. This coelution can affect the $R_{ac/coum}$ ratio of aged wines, and might lead to false results when classifying the wine variety [16].

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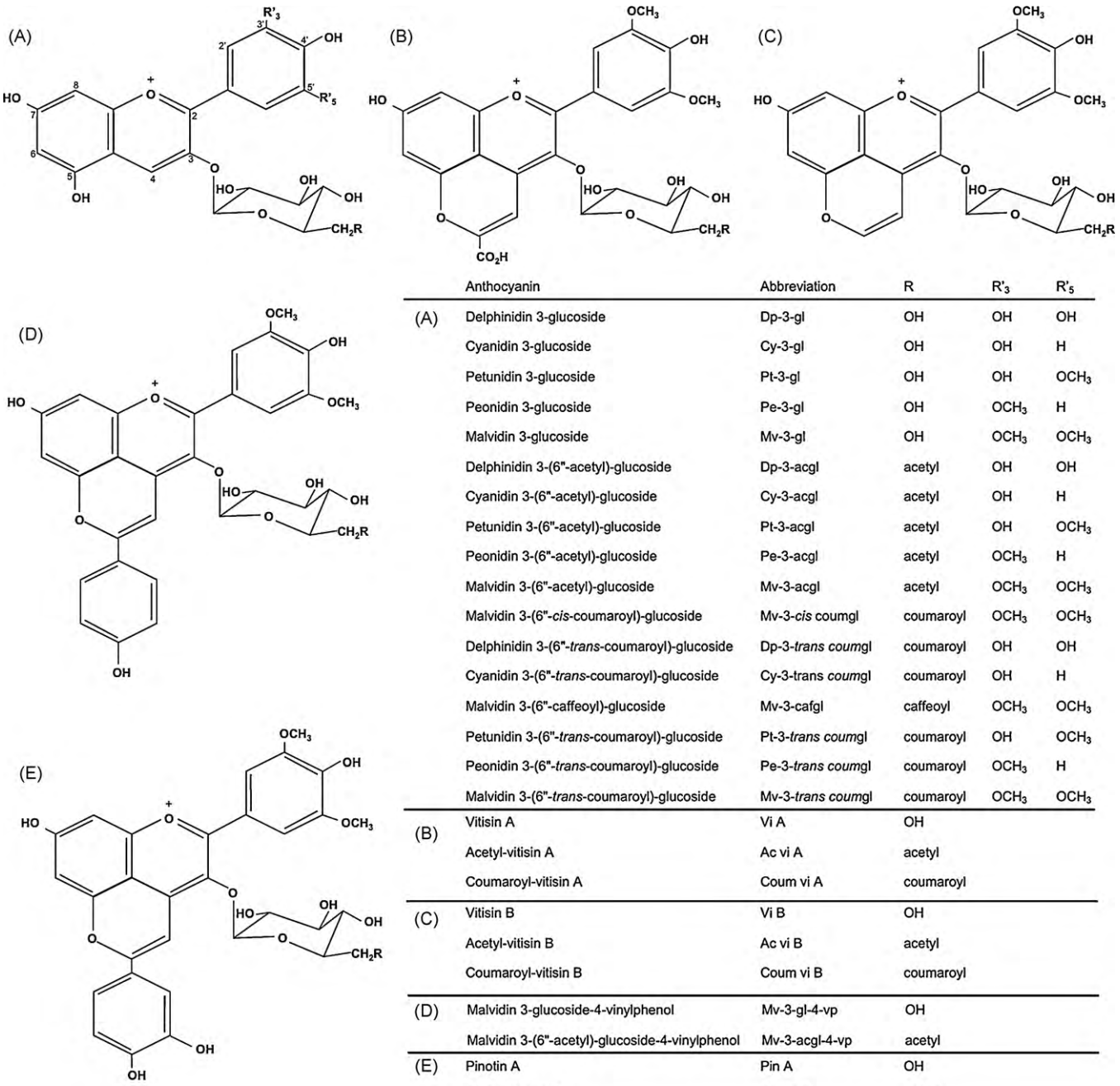

	Anthocyanin	Abbreviation	R	R'3	R'5
(A)	Delphinidin 3-glucoside	Dp-3-gl	OH	OH	OH
	Cyanidin 3-glucoside	Cy-3-gl	OH	OH	H
	Petunidin 3-glucoside	Pt-3-gl	OH	OH	OCH_3
	Peonidin 3-glucoside	Pe-3-gl	OH	OCH_3	H
	Malvidin 3-glucoside	Mv-3-gl	OH	OCH_3	OCH_3
	Delphinidin 3-(6"-acetyl)-glucoside	Dp-3-acgl	acetyl	OH	OH
	Cyanidin 3-(6"-acetyl)-glucoside	Cy-3-acgl	acetyl	OH	H
	Petunidin 3-(6"-acetyl)-glucoside	Pt-3-acgl	acetyl	OH	OCH_3
	Peonidin 3-(6"-acetyl)-glucoside	Pe-3-acgl	acetyl	OCH_3	H
	Malvidin 3-(6"-acetyl)-glucoside	Mv-3-acgl	acetyl	OCH_3	OCH_3
	Malvidin 3-(6"-*cis*-coumaroyl)-glucoside	Mv-3-*cis* coumgl	coumaroyl	OCH_3	OCH_3
	Delphinidin 3-(6"-*trans*-coumaroyl)-glucoside	Dp-3-*trans coum*gl	coumaroyl	OH	OH
	Cyanidin 3-(6"-*trans*-coumaroyl)-glucoside	Cy-3-trans *coum*gl	coumaroyl	OH	H
	Malvidin 3-(6"-caffeoyl)-glucoside	Mv-3-cafgl	caffeoyl	OCH_3	OCH_3
	Petunidin 3-(6"-*trans*-coumaroyl)-glucoside	Pt-3-*trans coum*gl	coumaroyl	OH	OCH_3
	Peonidin 3-(6"-*trans*-coumaroyl)-glucoside	Pe-3-*trans coum*gl	coumaroyl	OCH_3	H
	Malvidin 3-(6"-*trans*-coumaroyl)-glucoside	Mv-3-*trans coum*gl	coumaroyl	OCH_3	OCH_3
(B)	Vitisin A	Vi A	OH		
	Acetyl-vitisin A	Ac vi A	acetyl		
	Coumaroyl-vitisin A	Coum vi A	coumaroyl		
(C)	Vitisin B	Vi B	OH		
	Acetyl-vitisin B	Ac vi B	acetyl		
	Coumaroyl-vitisin B	Coum vi B	coumaroyl		
(D)	Malvidin 3-glucoside-4-vinylphenol	Mv-3-gl-4-vp	OH		
	Malvidin 3-(6"-acetyl)-glucoside-4-vinylphenol	Mv-3-acgl-4-vp	acetyl		
(E)	Pinotin A	Pin A	OH		
	Acetyl-pinotin A	Ac pin A	acetyl		

Fig. 1. Chemical formulae of some anthocyanins and pyranoanthocyanins in wine.

Even when it has already been established that high efficiency and resolution in chromatographic separation of wine anthocyanins are essential for the correct varietal classification of wine [16], achieving the highest selectivity in the separation remains relevant in order to separate and identify the highest number of compounds. McCallum et al. [17] published the use of a novel mixed-mode ion-exchange reversed-phase column to separate anthocyanin compounds extracted from grapes of *Vitis labrusca*, which also contain diglucosylated anthocyanins [18].

The Primesep B stationary phase is constituted by a basic group with positive charge embedded into a hydrophobic chain [19]. A different selectivity of this stationary phase is achieved with the combined mechanisms of reverse chromatography and ion exclusion chromatography based on the Donnan exclusion phenomena [20]. In these phenomena, ions of opposite charge to that of the fixed ions are attracted by electrostatic forces, while those of the same charge type are repelled and thus excluded [20]. The anthocyanins in the highly acidic media of mobile phase are mainly in the cation flavylium form [1], while the formate counter ion neutralizes the positive charge embedded in the hydrophobic chain of stationary phase. Under this condition, anthocyanin retention would be governed by their hydrophobic nature. The different selectivity of this stationary phase could be an interesting solution in the complicated separation of anthocyanins and pyroanthocyanins in wine.

In the present work, separation of anthocyanins and pyranoanthocyanins in young and aged Cabernet Sauvignon wines and other varieties is evaluated using a mixed-mode column (Primesep B2, Prospect Heights, USA). The results are compared with those obtained with a high efficiency C-18 column of the same dimen-