Are pyranoanthocyanins involved in sensory effect in red wines?

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Abstract. During the wine-making process and ageing, the formation of various groups of pyranoanthocyanins leads to enhance and to stabilize the red wine colour. Although their chemical properties and their formation kinetics have been well-described, data about their sensory impact are scarce. Micro-oxygenation can be used to favor the formation of pyranoanthocyanins and also to improve wine quality. For this study, both analytical and sensory aspects were used. Purification steps applied on an oxidized red wine lead to characterize three new original compounds. Sensory analysis was declined into two aspects. First the purification of an A-type vitisin-rich fraction will be used for evaluating the possible sensory impact of pyranoanthocyanins; then the correlation between the tasting of micro-oxygenated red wines and the concentrations of these wine samples in pyranoanthocyanins will determine if these pigments could enhance aroma characteristics.

1. Introduction

The study of pyranoanthocyanins, their chemical structure, their formation kinetics and their contents in red wines provides crucial information about the wine evolution and especially the color stability. Pyranoanthocyanins are the result of a nucleophilic substitution in C-4 position on the anthocyanin moiety, which forms an additional ring D between the OH group at C-5 and the C-4 position of the anthocyanin pyranic ring [1]. Anthocyanins, due to their high reactivity in aqueous media, can easily react with pyruvic acid to form A-type vitisins [2,3]; with ethanal to form B-type vitisins [4]; with vinyl compounds to form phenyl-pyranoanthocyanins or pinotins [5], [6]; or with 8-vinylflavanol oligomers to form flavanol-pyranoanthocyanins [7,8].

Micro-oxygenation (MOX), which consists of adding low quantities of oxygen at different stages of the wine-making process or ageing, also contributes to enhance and to stabilize red wine color through the formation of pyranoanthocyanins, for the cycloaddition of anthocyanins requires an oxidation step.

Their chemical properties and their formation kinetics have been studying for over 20 years; however scientific data about their sensory data are scarce. This study had two purposes: the first aim was to fractionate an oxidized red wine in order to purify and characterize new pigments; the second one was to evaluate the contribution of pyranoanthocyanins in sensory aspects.

2. Materials and methods

2.1. Chemicals

Deionized water was purified with a milli-Q water system (Millipore, Bedford, MA). Methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). Formic acid and trifluoroacetic acid were purchased from Sigma Aldrich (Saint-Louis, MO).

2.2. Fractionation and purification of an oxygenated red wine

2.2.1. Wine sample

The studied wine was a young Merlot red wine brought from Rauzan cooperative winery (Bordeaux region, France) aged in stainless steel tanks for 12 months [pH3.5, alcohol 12%vol.]. This sample was previously taken in variable oxygenation and acidity conditions during 4 months before being stored in controlled temperature during 4 other months. The modality used was: wine sample placed at pH3 and oxygenated with an oxygen rate of 20 mg/L.

2.2.2. Fractionation strategy

The sample of Merlot red wine was first fractionated onto a 530 × 65 mm i.d. Amberlite® XAD 16N column. The column was first washed with milli-Q water to elute sugars and organic acid such as tartaric acid. Wine sample was eluted using acetone acidified with 1% formic acid (HCOOH), evaporated, re-solubilized and lyophilized.

Then, centrifugal partition chromatography (CPC) was applied on the obtained wine powder. The system

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used was a gradient elution in ascending mode [9]. Ethyl acetate, 1-butanol and water acidified with 0.1% trifluoroacetic acid (TFA) were used for each phase of the solvent system, in the following proportions: stationary phase (5:5:90); initial mobile phase (77:15:8); and final mobile phase (40:46:14). The flowrate was 15 mL/min. The sample was dissolved with the same quantity of stationary phase and initial mobile phase. The first block (Block 1) was collected from 0 to 35 min, and the second block (Block 2) was collected from 35 to 70 min. Blocks 1 and 2 were evaporated and lyophilized.

2.2.3. Purification and pigment characterization

Pigments from blocks 1 and 2 were purified by preparative and semi-preparative HPLC. Each collected pigment was then analyzed and fragmented using UHPLC-ESI-Q-TOF system. The UHPLC *Agilent 1290 Infinity* series consisted of a quaternary pump, a solvent degasser, an autosampler and a diode-array detector (DAD). The analysis was carried out on a 2.1 × 50 mm i.d., 1.8 μm *Eclipse Plus* C18 column. The UHPLC system was coupled to an ESI-Q-TOF-MS *Agilent 6530 Series Accurate* quadripole time-of-flight (Q-TOF) with an *Agilent Jet Stream Technology* ESI. The mass spectrometer was operating in extended dynamic range of 2 GHz with the mass range up to 1700 Th, with a drying gas flow and temperature respectively set at 9 L/min and 300 °C. The capillary voltage was 4 kV. The software used for the determination of the exact molecular formula of the trimeric anthocyanins was MassHunter Qualitative Analysis. The solvents used for the gradient were A: H2O/HCOOH (999:1) and B: MeOH/HCOOH (999:1). The gradient consisted of: 30–80% B in 9 min, 80–100% B in 0.5 min at a flow rate of 0.4 mL/min. The column was washed with 100% B for 2 min and re-equilibrated with the initial conditions for 2 min.

2.3. Assay on pyranoanthocyanins sensory aspect

2.3.1. Molecular approach: purification of an A-type vitisins-rich fraction

It was interesting to focus our attention on the possible sensory contribution of pyranoanthocyanins by tasting fractions of such pigments. The last CPC fraction of the Merlot oxygenated red wine, corresponding to the wash block, was fractionated with two purification steps. First, preparative HPLC was applied on this sample to collect each pigment separately. Pre-purification was carried out on a 21 × 250 mm i.d., 5 μm *Nucleosil* C18 column. The HPLC system was *Varian Prostar* series, coupled with a *Prostar 325* detector. The solvents used were: solvent A: H_2O/TFA (999:1) and B: Acetonitrile/TFA (999:1). The gradient consisted of 10-45% B in 20.5 min, and 45–100% B in 1 min at a flow rate of 12 mL/min. The column was washed with 100% B for 7 min and then stabilized with the initial conditions for another 7 min. Then, a semi-preparative step was applied on each collected pigment using the HPLC system described above. Purification was carried out on a 8 × 250 mm i.d., 5 μm *Prontosil* C18 column. The solvents used were A: H_2O/TFA (999:1) and B: Acetonitrile/TFA (999:1). The gradient consisted of 10–20% B in 10 min, 20–30% B in 10 min, 30–60% B in 10 min, 60–100% B in 1 min at a flow rate of 3 mL/min. The column was washed with 100% B for 7 min and then stabilized in the initial conditions for another 7 min.

The purity was estimated at 280 and 520 nm using the UHPLC system described above. The analysis was carried out on a 2.1 × 50 mm i.d., 1.8 μm *Eclipse Plus* C18 column. The solvents were A: H_2O/HCOOH (999:1) and B: Acetonitrile/HCOOH (999:1). The gradient consisted of 10–70% B in 10 min, 70–100% B in 0.5 min at a flow rate of 0.4 mL/min. The column was washed with 100% B during 1.5 min and re-equilibrated with the initial conditions for 2 min.

Table 1. Modalities of the micro-oxygenation treatment.

Timing MOX	Date	Rate (mL/L/month)	Time of treatment (days)
Post-fermentation maceration	17/10/01 – 25/10/01	60	9
Before MLF	26/10/01 – 29/10/01	20	4
	29/10/01 – 30/10-01	10	1
MLF	30/10/01 – 29/11/01	0	–
Ageing	29/11/01 – 6/12/01	5	8
	6/12/01 – 20/12/01	3	15
		TOTAL	23.8 mL O_2 /L wine

2.3.2. Wine-tasting

A technical wine-tasting was applied on micro-oxygenated Saint-Émilion red wines with ageing potential from 2001 vintage. Modalities of the MOX treatment was summarized in the following table (Table 1). Each control and MOX red wines were distributed in two batches and aged either in oak barrels during 18 months, or in stainless steel during 9 months, before bottling.

The panel included 11 people in the laboratory and 2 technical engineers from the French Institute of Vine and Wine (Pôle IVF Bordeaux-Aquitaine). All tasters were informed that the wines had been micro-oxygenated and aged in different containers. Each wine sample was presented in a black glass and encoded with randomly-selected 3-digit numbers. Wines were evaluated on a predefined score sheet that included 12 descriptors in two categories: olfactory attributes (intensity, fresh and cooked fruits, floral, vegetal, spicy, and woody), and 'in mouth' attributes (acidity, bitterness, astringency, tannins' smooth and long-tasting aromas). The sensory characteristics had to be rated on a non-anchored rating scale of 10 cm from weak to intense as reference points.

One-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) *post hoc* test with statistical difference at $p < 0.05$ was carried out using Statistica V.10 (Statsoft Inc., Tulsa, OK, USA).

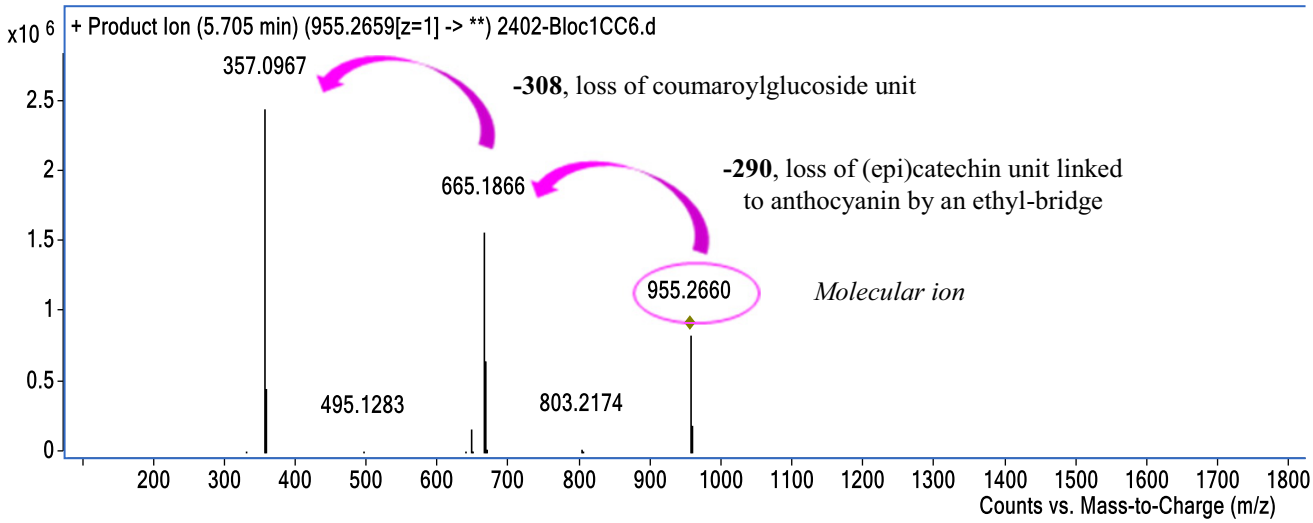

Figure 1. MS/MS fragmentation of pigment 2 (Block 1).

Table 2. Pigment characterization according to CPC blocks.

Block	Pigment	Mass m/z	ppm	Formula	Name
1	1	663.1708	−1.35	$C_{34}H_{31}O_{14}^+$	Vit.B Mv3cmG
	2	955.2655	0.52	$C_{49}H_{47}O_{20}^+$	Mv3cmG-ethyl-cat
	3	951.2342	0.63	$C_{49}H_{43}O_{20}^+$	PyrMv3cmG-(epi)cat
	4	805.1974	−1.49	$C_{40}H_{37}O_{18}^+$	PyrMv3G-(epi)cat
2	5	1135.2714	−0.51	$C_{57}H_{51}O_{25}^+$	PyrMv3acG-procDim
	6a	779.2182	2.31	$C_{39}H_{39}O_{17}^+$	Pn3G-ethyl-(epi)cat
	6b	1093.2608	3.58	$C_{55}H_{49}O_{24}^+$	PyrMv3G-procDim
	7	707.1607	4.8	$C_3H_{31}O_{16}^+$	Vit.A Mv3cmg
	8	791.1818	0.77	$C_{39}H_{35}O_{18}^+$	PyrPt3G-(epi)cat
	9	775.1869	1	$C_{39}H_{35}O_{17}^+$	PyrPn3G-(epi)cat
	10	805.1974	−2.51	$C_{40}H_{37}O_{18}^+$	PyrMv3G-cat
	11	805.1974	−2.42	$C_{40}H_{37}O_{18}^+$	PyrMv3g-epicat

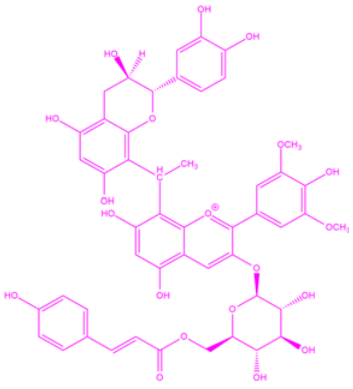

Figure 2. Structure of pigment 2 (Block 1): malvidin-3-*O*-coumaroylglucoside-ethyl-catechin.

3. Results

3.1. Purification and characterization of new pigment structures: Blocks 1 and 2

Four pigments from Block 1 and eight pigments from Block 2 were characterized. Each pigment was numbered and listed in the following table (Table 2). Block 1 was essentially composed of coumaroylated pigments. Attention was focused on pigment n°2 especially, which had a theoretical mass of 955.2655. After MS/MS fragmentation, the result was the fragmented ions 665 and 357 (Fig. 1), 665 corresponding to the loss of an (epi)-catechin unit linked to an anthocyanin by an ethyl-bridge (−290), 357 corresponding to the loss of a coumaroylglucoside unit (−308). The molecular structure was also validated by nuclear magnetic resonance (NMR – data not shown) for the pigment was collected in sufficient quantity (at least 0.5 mg). This pigment was a new original structure of malvidin-3-*O*-coumaroylglucoside-ethyl-catechin (Fig. 2).

About Block 2, attention was focused on pigments 5 and 8. Pigment 5 had a theoretical mass of 1135.2714; pigment 8 a mass of 791.1818. MS/MS fragmentation and determination of their respective formulas (Table 2) showed two potential new original structures: pyranomalvidin-3-*O*-acetylglucoside-procyanidin dimer and pyranopetunidin-3-*O*-glucoside-(epi)catechin. NMR data are also required to validate this hypothesis (in process).

3.2. Purification of the A-type vitisins-rich fraction

A great quantity of molecules is required to such a wine-tasting assay, and also the first purification step is in process. After a first run with preparative HPLC, the collected pigments were pooled together for analysis by UHPLC Q-TOF. Six pigments were characterized, all of them being an A-type vitisins (Fig. 3). Pigments (from a to f) were listed and characterized in the following (Table 3).

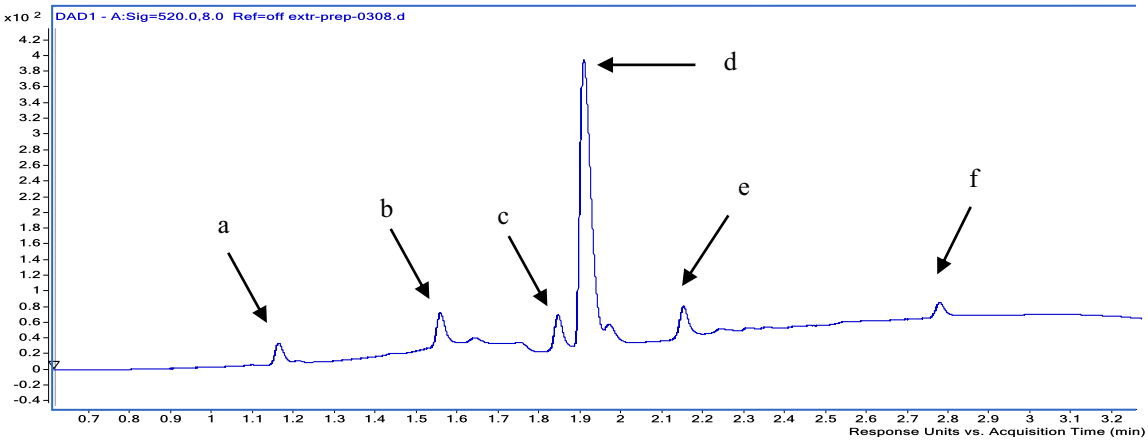

Figure 3. Chromatographic profile at 520 nm.

Pigment	Mass m/z	ppm	Name
a	533.0926	–4.57	Vit.A Dp3G
b	547.1082	–3.61	Vit.A Pt3G
c	531.1133	–3.9	Vit.A Pn3G
d	561.1239	–5.09	Vit.A Mv3G
e	603.1344	–3.33	Vit.A Mv3acG
f	707.1607	–3.7	Vit.A Mv3cmG

Table 3. Identification of pigments from the CPC wash fraction after first preparative HPLC.

A second purification step is required to obtain at least 80–85% of purity before a technical tasting.

3.3. Sensorial analysis of the micro-oxygenated wines

The evaluation of the sensory parameters showed that MOX wines were more appreciated than the control ones. Significant differences were found for the vegetal and woody criteria. MOX wines aged in stainless steels were judged more vegetal than the other modalities, but this result was also found during all the tastings organized during the wine-making process, highlighting a possible lack of maturity for harvesting. Then, MOX wines aged in oak barrels and their associated control ones were found woodier than the others, with a stronger astringency and less tannins' smooth. For the other characteristics, the results showed that MOX wines were more appreciated and evaluated than the control ones: MOX wines aged in stainless steels were judged less acidic, astringent and bitter; those aged in oak barrels were more fruity, floral and spicy, with more astringency and long-tasting aromas.

It is well-known that high-quality red wines are traditionally aged in oak barrels during ageing [10]. Indeed, oak permeability improves natural oxygen permeation (between 1.66 and 2.5 mL/L/month) [11] which leads to an increase of the color intensity and stability. A MOX treatment could also be used during oak ageing to accelerate red wine maturation [12]. To confirm this tendency, a characterization and quantification of anthocyanins and derived-pigments, pyranoanthocyanins in particular, will be established (in process). Following these results, further statistical analysis will highlight correlations between the results of the wine-tasting and the concentrations in pyranoanthocyanins.

4. Conclusion

For the first part of the experiment, three original structures were highlighted, and one was already fully characterized. NMR analysis will determine the structure of the two other molecules and quantification will determine if there are differences between non-oxygenated and oxygenated red wines.

This study will also attempt to answer the question of the sensory impact of pyranoanthocyanins and to clarify their possible correlation towards organoleptic characteristics. The planned results in the months to come, through the purification of an A-type visitin-rich fraction, and the quantification of pyranoanthocyanins associated with the red wine tasting, will be decisive to determine if pyranoanthocyanins are significantly involved in such a role.

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