FLANAN-3-OL COMPOSITIONAL CHANGES IN RED GRAPE BERRIES (*VITIS VINIFERA* L. CV CABERNET FRANC) FROM TWO TERROIRS OF THE LOIRE VALLEY (FRANCE)

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Key words: Tannins, maturation, Vitis vinifera var. cabernet franc, skin, seed, histochemistry

Abstract

The quantity and the quality of flavonoïds are important parts of the global quality of the grape berries. Especially, the tannins are responsible of some majors flavour properties of the red wines including colour, bitterness and astringency. Nevertheless, their synthesis and properties are still misunderstood. Thus, the comprehension of the relations between environment and setting of this tannic pool, up to the harvest of the grapes, is not sufficient. The tannin composition was monitored since the middle of the first growth period (30 days after the onset of flowering) to the average maturity, for two plots. The study the stage of the berries, and not the average value of the parameters. Thus, the contribution of a more functional relation between composition and physiological stage of the grape constitutes a powerful lever for interpretation. The composition in flavan-3-ols and in proanthocyanidins of skins were determined by HPLC reversed phase and by histochemistry. The linking of these two analytical techniques allowed the association of quantitative and spatial data. This original approach pointed out the importance of the period previous maturation in relation with the stage of maturation but also others factors, such as the number of seeds. There was little evolution of the tannins after veraison. It is suggested that the tannic pool is set before veraison. More, a relation between the number of seeds and the quality of the berries was shown.

Résumé

La quantité et la qualité des flavonoïdes sont des éléments importants de la qualité de la baie. En particulier, les tannins contribuent de manière essentielle aux propriétés spécifiques des vins rouges telles que la couleur, l'astringence et l'amertume. Cependant, leur synthèse et leurs propriétés sont encore mal connues. Ainsi, la compréhension des relations qui existent entre, d'une part, le milieu et d'autre part la mise en place de ce pool tannique jusqu'à la vendange est insuffisante. La composition en tannins des pellicules est suivie depuis le milieu de la phase de croissance herbacée (30 jours après le début floraison) et jusqu'à une maturité normale, sur deux parcelles. L'étude considère un stade donné de développement et non pas la valeur moyenne des paramètres à la parcelle. Ainsi, l'apport d'une relation plus fonctionnelle entre composition et stade physiologique du raisin constitue un levier puissant d'interprétation. La composition en flavan-3-ols et en proanthocyanidines des pellicules est déterminée par HPLC-phase inverse et par histochimie. Le couplage de ces deux techniques permet d'associer des informations quantitatives et spatiales. Cette approche originale permet de mettre en évidence l'importance de la période pré-maturation, en relation avec l'avancement de la maturation mais également d'autres facteurs, comme le nombre de pépins. Qualitativement et quantitativement, les teneurs évoluent peu après véraison. Il est suggéré que le pool tannique est acquis avant véraison. D'autre part, il est montré une relation entre le nombre de pépins des baies et leur qualité.

Introduction

The phenolic composition of the grape at maturity is a major information for the winemaker. These compounds -anthocyanins, flavonols and procyanidins- are important for the quality of the products, especially for the red wine, because they are responsible for their bitter, astringency and colour properties (BROSSAUD *and al.*, 2001 ; CHEYNIER *and al.*, 1999 ; HASLAM, 1999, 1980 ; NOBLE, 1999 ; SINGLETON and TROUSDALE, 1992 ; ARNOLD *and al.*, 1980 ; LEA and ARNOLD, 1978). Many environmentals and culturals factors can affect the accumulation of these compounds such as water status, nitrogen, temperature (DELGADO *and al.*, 2004, DELOIRE *and al.*, 2003 ; OJEDA *and al.*, 2002 ; MATTHEWS *and al.*, 1987). Beside the level on phenolics compounds, it is important to approach their molecular structure, and also their ability to extraction, which allows the passage in the must during the wine process (GLORIES, 1998). Thus, the phenolic maturity can be defined like allowing simultaneously the obtention of a high level in the grape and a good diffusing capacity into the wine.

However, if the tannins are major compounds of the grape, their synthesis, properties, and availability are still badly known.

The studies carried out in INRA in Angers (mid Loire valley, France) on *Vitis vinifera* var. cabernet franc showed that there are relations between the type of soils and the polyphenolic composition of the berries and the wines (BROSSAUD, 1999 ; ASSELIN *and al*., 1992). Concerning the relative contents of flavan-3-ols (tannins) for a known origin, the evolution is not visible in the month preceding harvest, and this, as well for the seeds as for the skins (BROSSAUD, 1999).

In agreement with other studies (KENNEDY, 2000), it seems that the differences between plots could be observed before ripening. Moreover, by only considering the average of the sampling from the plot, and not an homogeneous stage, it is probable that differences between samplings are masked. Lastly, the analytical techniques employed limit our possibilities of investigation, since for example it is possible to know only the average degree of polymerization of tannins (Dpm).

In that, we thought that it was important, for better knowing the relation between the terroirs and the polyphenolic quality of the grapes -the phenolic maturity- to study the early installation of the polyphenolic pool before ripening and to allows spatialization of the tannins in cellular tissue by coupling the biochemical and histochemistry approaches.

Matérials and méthods :

Planting material : two parcels were selected among a network of 14 experimental plots which had been monitored since 2002. The plots are led in an identical way (with the plant health treatments near). They are located in the middle Loire Valley area, between Saumur and Bourgueil, on sedimentary terrains from the secondary and tertiary eras. Their main characteristics are given in table n°1.

Soils descriptions : each plot is homogeneous from a geo-pedological point of view. The soils profiles were carried out by observation and analyses, with the use of an auger. These profiles are given on figure n°1.

Water status : leaf water potentials were measured 22, 51 and 69 days after mid-flowering (the last, 8 days after mid-veraison) after at least 10 days without rainfall and using a pressure chamber ; water stress with $\Delta C13$ discrimination (veraison-maturity). Some informations about the grapevine behaviour and spatial land distribution of soil were collected by electric resistivity measurements (GOULET, 2004).

Climatic data were obtained with a Campbell meteorological station (humidity, sunshine, temperature). Potential evapotranspiration (ETP) has been calculated from the Penman-Monteith equation (MONTEITH, 1965). The heliothermal parameter index was calculated according to HUGLIN (HUGLIN, 1978).

Sampling : the sampling of berries, with pedicel, were carried out by two people. In order to sample homogeneous berries in term of stage of development, flowers were marked at the stage “falls of the floral cap” (600 flowers per plot). At the beginning of ripening, the collected berries were sorted out by rheology. Finally, during the ripening, the berries were sorted by density. The studied stages

were : flowering +31, 41 and 51 days after mid-flowering (150 marked berries for each stage), veraison (400 berries), end veraison (when 100% of the berries turned red ; probable alcoholic degree "DAP" # 6-7), end ripening (DAP > 11.5). In the two last stages, 700 berries were taken. These 700 berries were sorted by immersion in baths of decreasing densities (water-NaCl), then washed immediately with water and dried. It was therefore possible to obtain homogeneous classes of berries.

Classical analyses : the methods were as follows. On filtered juice ; malic acid : enzymatic method ; tartaric acid : metavanadate ; total sugars : differential refractométry ; total acidity : titrimetry.

Analyticals of the flavonoïds were carried out after methanolic and acetonic extraction of crushed skins, (crushing with a mixer mill RETSCH MM301). Separations and analyses (HPLC) : tannins, after fractionation on gel TOYOPEARL® HW40 and thiolysis, according to conditions described by BROSSAUD (BROSSAUD, 1999) and LABARBE (LABARBE, 2000), on a KONTRON® 400 chain and a UV-Vis. detector.

For measurements by histochemistry, the preparation took place directly in the field in order to obtain in fine 5 berries representing the median class of the plot. We took 0,25cm^2 of skin on each face of berries and fixed with glutaraldehyde. The samples were freezed (ethanol) then included in resin TECHNOVIT 7100 as described by KROES (KROES, 1998). The section, from 1,5µm were obtained by a microtome LEICA RM 2165. Staining was according to the DMACA method (GUTMANN, 1991). The observations were carried out with an OLYMPUS BH-RFC microscope coupled to a 3CCD SONY camera. The counting of cells containing phenolics was realised on three sections for each berry, taken randomly on the blade. For each section, 3 portions were counted. The image analysis was processing with the NIH Image program for Macintosh (National Institute of Mental Health, U.S.A.). The images were analysed from the red channel and after conversion in grey scale (256 levels).

Results and Discussion

Environment :

Climatic data : The year 2003 can be regarded as exceptional for the area, in particular from the point of view of the sunshine and the temperatures. On the other hand, rainfalls were normal (Table n°2). The heliothermal index was thus very high (1650-1750 in "average" year). Finally the ETP was very high too.

Soils : M2 is an sandy-clay soil of the Senonien. M3 consists of sands (alluvia of the Loire river). The soil water supply (RU) calculated on M3 is low, contrary to M2. The profiles obtained by electric resistivity measurements (figure n°2) show that M2 is not very resistant on all the profile and in particular below 80 cm, corresponding to the horizon "SA". The profile evolves little between the field water capacity (in April 2002) and the dry period (in August 2003). On the other hand the profile of M3 is quite different : the electric resistivity values are clearly higher, and moreover, in August 2003, they evolved (in depth), being more resistant.

Water status : Measurements began approximately 20 days after flowering, with the objective to measure the leaf water potential every 10 to 15 days. Unfortunately, an inappropriate storm prevented one measurement (43 days after mid-flowering). Thus, three measurement were practised before ripening : 22, 51 and 69 days after mid-flowering (results are shown in figure n°3). We can see that the plots were not water-stressed. After ripening, measurements of water stress through $\Delta C13$ discrimination, showed no hydrous constraint for M2 and an average level of constraint for M3. For the period of maturation results are showed in table n°2.. In 2003, 3 rainy days occurred at regular intervals, at F+28, F+43 and F+54 days, whereas the hydrous balance was still largely positive for M2 and slightly for M3. On the other hand, after the veraison, the balance regularly and strongly worsened until 93 days after mid-flowering.

Grape development :

Histological observations of the berry skin, stained with DMACA show two type of cells : cells without tannins, and cells coloured (figure n°4). We can see different types of coloured cells, according to others studies (AMRANI-JOUTEI *and al.,* 1994), from cells with uniform coloration to cells with spherical or shapeless inclusions, free or stucked to the tonoplaste.

Date effect : before veraison : according to table n°4, the date effect is significant only for the modality "Mean intensity of the coloured zones". This can be interpreted by the fact that the average intensity of the slides was identical but that the tannins was more coloured. We can think that tannins occupied less space. They would be more aggregated. Concerning the biochemistry analyses of tannins, quantity increased before ripening, for the two plots, afterwards stabilized before decreasing quickly at the beginning of veraison and then stabilizing around maturity (table n°3). This is overall in agreement with other studies (DOWNEY et al., 2003; KENNEDY et al., 2001; FREITAS and GLORIES, 1999; CZOCHANSKA et al., 1979). However, the increase in the size of berries during first growth period involves that the content of tannins, per kg of berries, does not evolve. Then, if we observe a tendency to the decreasing during maturation, it remains weak. The results concerning the mean degree of polymerisation, or the proportion of epigallocatechin (% prodelphinidins) are in agreement with previous studies (BROSSAUD, 1999).

Site effect : It is highly significant before ripening (table n°4). The contents of tannins for M3 were weaker, whatever the sampling dates (table n° 3 and n°4). Differences were well marked before ripening, in spite of no difference in precocity or water status. The proportions of prodelphinidins (table n°3) were less important in M3.

Tannins and heterogeneity : The values for the most sweet berries (In table n°3, the last 2 stages) show that the tannins contents are the weaker, for the two sites, even expressed in mg/kg of berries, however the ratio square/volume is more advantageous. This lower content, whereas the evolutions are weak at the maturity could not thus be explained only by a higher level of maturity. These berries (with a smaller volume and having less seeds), seem to have a different behaviour, which cannot be explained by a more advanced maturity.

Conclusion

This study shows the importance of the period previous ripening in the synthesis of tannins in the skin. The differences between the two terroirs are preserved during the maturation, but it's is particularly true until the installation of the biosynthesis of the anthocyanins. The water status of the two plots are different after veraison, but can not explain that M3 is less rich in tannins.

This study shows that the linking of two analytical techniques (histochemistry and biochemistry) allowed the association of quantitative and spatial data. In particular, the evolution of the aggregation of the tannins can be highlighted and be connected to an average composition. This approach must be extend to the end of the maturation.

This study shows that the measurement of the heterogeneity of the sampling is important to appreciate the quality of the product. The number of the seeds which influence the size of the berries before veraison, is to be taken into account, during maturation, in particular for better appreciating and understanding the heterogeneity of a sampling.

This study show lastly that with the same precocity and water status, two different type of soils could produce very different type of berries, in particular in term of tannins composition.

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List of tables and figures

Figure n° 3 : Leaf water potential for the network. M2 and M3 are compared with 12 others plots. These 14 plots represent more than 75 % of the soils met in the area. M2 and M3 results are shown in relief.

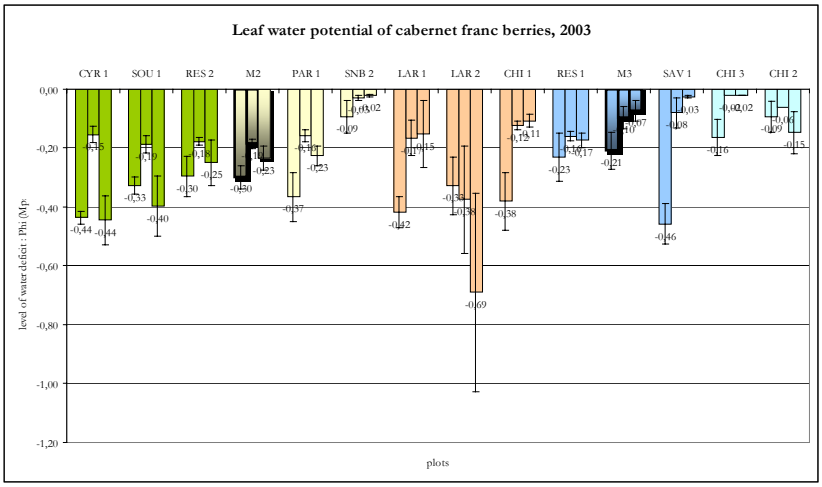


Figure N° 1 : soils profiles. SL = sandy silt ; S = sand ; SA= sandy clay ; Sgr = gravely sand ; RU = soil water supply

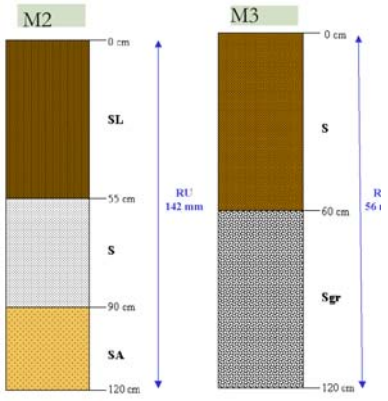


Figure n° 4. Observation of the berrie skin by histochemistry.
Plot M2, 31 days after flowering, non-exposed face.
Thin section of skin stained with DMACA.

a. cells without any coloration
b. cells with uniform coloration
c. cells with fine granulations homogeneously distributed into the vacuole
d. cells with small spherical inclusions, some of them stuck to the tonoplast
e. cells with large round and distorted inclusions, free inside the vacuole

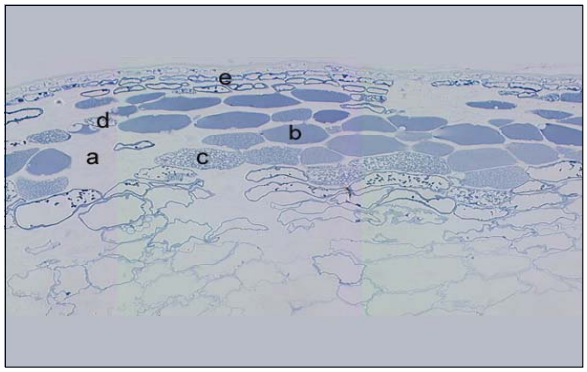
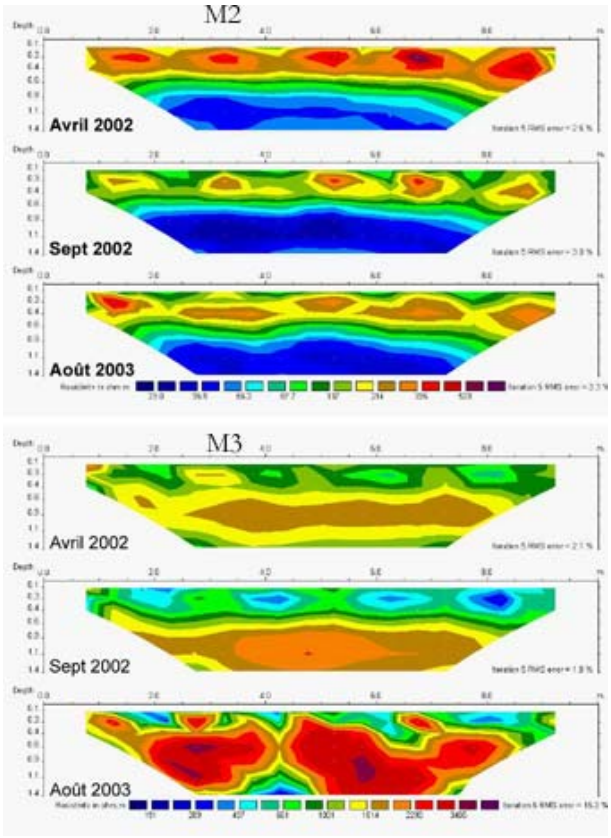


Figure n° 2 : Grapevine behaviour and spatial land distribution of soil by electric resistivity measurements. Avril = april ; Août = august



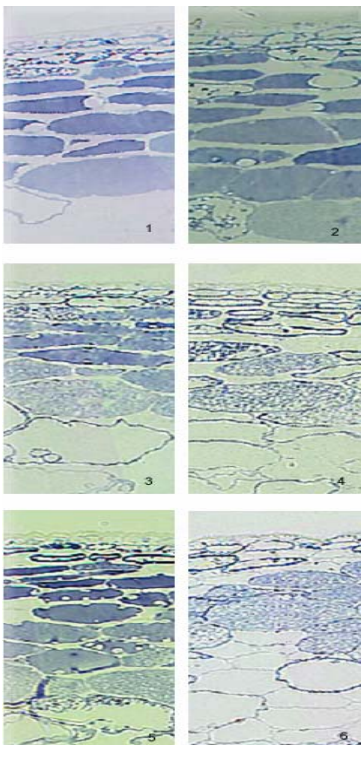


Figure n°5 : Thin section of skin stained with DMACA, from two plots and for three dates.

Legend :

1. M2 - 31 days after mid-flowering
2. M3 - 31 days after mid-flowering
3. M2 - 41 days after mid-flowering
4. M3 - 41 days after mid-flowering
5. M2 - 51 days after mid-flowering
6. M3 - 51 days after mid-flowering

Table n° 1 : Main characteristics of the experimental plots.

Code	M2	M3
Name plot	SOU2	SNB1
Cultivar	Cabernet franc, clone 210	Cabernet franc, clone 210
rootstock	3309C	3309C
average age in 2003	18 years old	19 years old
Square	192	235
Density	1,90*1,05 m	2,00*1,05 m
Trellising	vertical	vertical
Pruning	Guyot (simple)	Guyot (simple)
Weeding	chemical	chemical

Table n° 2 : 2003 climatic data and hydrous constraint during ripening (ΔC13)

April - september	M2	M3
Humidity (%)	66,4	72,1
Rainfall (mm)	313	303
Température (S in °C)	3338	3432
Sunshine (S, in W/m2)	989000	1019000
ETP (mm)	995	1054
Heliothermal index	2167	2272
Hydrous constraint during ripening		
DC13 ($^{0}/_{00}$)	-26,58	-23,73

Table n° 4 : Statistical analysis for the histochemical data.

mean intensity of the slide (1) :

Multiple tests of comparisons for the variable "date" :
(Newman-Keuls) confidence interval : 95%
Classification and regroupings of the groups not significantly different:

Modalities	Mean (%) *	groups
31 days after flowering	14,767	A
41 days after flowering	14,761	A
51 days after flowering	14,425	A

Multiple tests of comparisons for the variable "plot" :
(Newman-Keuls) confidence interval : 95%
Classification and regroupings of the groups not significantly different:

Modalities	Mean (%) *	groups	
SOU2	17,564	A	
SNB1	11,738		B

Mean intensity of the coloured zones (2) :

Multiple tests of comparisons for the variable "date" :
(Newman-Keuls) confidence interval : 95%
Classification and regroupings of the groups not significantly different:

Modalities	Mean (%) *	groups		
31 days after flowering	29,087			C
41 days after flowering	30,872		B	
51 days after flowering	38,218	A		

Multiple tests of comparisons for the variable "plot" :
(Newman-Keuls) confidence interval : 95%
Classification and regroupings of the groups not significantly different:

Modalities	Mean (%) *	groups	
SOU2	33,462	A	
SNB1	27,290		B

* Mean (%) : 0 for white and 100 for black.
(1) : measurement of the mean level of blue colour, from the red channel (converted in 256 levels of greys) of the triCCD sensor for all the image (640*480 pixels).
(2) : measurement of the mean level of blue colour, from the red channel (converted in 256 levels of greys) of the triCCD sensor for all the tannins of the image (640*480 pixels).

Table n° 3 : Biochemical results for the two plots.

Plot	date : days after mid flowering	Stage	%of each class (in number of berries)	Weight of 1000 berries (g)	Weight seeds (g/kg of berries)	Number of seeds / 1000 berries	Sugars g/l	Weight of seed (mg)	Tannins g/kg berries	Tannins g/1000 berries	Mean degree of polym. (DPm)	% Prodel-phinidins
M2	31	first growth period		600	110,4	1620		40,7	1,36	0,81	17,0	38,0%
	41			749	83,6	1340		45,7	1,04	0,76	16,2	40,2%
	51			848	86,1	1360		51,2	1,47	1,19	19,0	45,1%
	59	Ver., firm		1086	97,6	2000		51,8	1,37	1,45	15,8	41,6%
		Ver., medium		1151	102,6	2200		53,2	1,22	1,39	16,9	43,2%
		Ver., soft		1134	86,7	1880		50,7	1,15	1,26	16,4	43,5%
	80	100 % of veraison (mid V+17)	51	1433	46,3	1300	192		0,99	1,43	18,8	49,2%
			23	1569			176		0,98	1,54	18,3	45,8%
			8	1371	59,8	1620	157		0,94	1,26	18,1	45,2%
			4	1245			142		1,75	2,15	19,3	47,1%
			14	918	84,8	1600	124		1,61	1,44	17,9	44,8%
	109	Harvest (mid V+48)	3	1145	38,1	1060	258		0,72	0,83	21,0	40,9%
			25	1275			240		0,87	1,05	21,2	45,4%
			48	1573	43,0	1505	225		0,65	1,02	18,5	41,0%
			16	1693			211		0,85	1,44	17,9	41,8%
			4	1522	50,6	1660	192		0,75	1,13	17,6	43,8%
			3	1439			165		0,91	1,37	20,2	41,9%
M3	31	first growth period		577	95,6	1260		41,0	1,42	0,77	18,4	32,1%
	41			710	78,7	1360		39,7	1,19	0,82	15,6	34,4%
	51			836	78,2	1480		41,9	1,11	0,93	15,8	36,7%
	55	Ver., firm		992	85,1	2020		44,6	1,31	1,39	15,8	36,0%
		Ver., medium		1018	81,8	1760		45,9	1,08	1,07	13,9	33,6%
		Ver., soft		1098	70,9	1600		45,1	1,04	1,06	15,9	34,9%
	74	100 % of veraison (mid V+15)	25	1114	45,1	1260	192		0,72	0,83	14,2	30,9%
			45	1231			176		0,90	1,27	16,1	38,6%
			14	1077	57,0	1400	157		0,95	1,03	15,5	35,0%
			7	786			142		1,09	0,89	15,8	35,7%
			10	685	63,3	1340	124		1,23	0,93	15,1	33,4%
	96	Harvest (mid V+37)	5	955	38,1	1080	251		0,67	0,68	14,7	32,0%
			30	1067			232		0,68	0,72	15,3	32,0%
			49	1320	46,2	1460	215		0,80	1,04	16,7	36,5%
			14	1611			202		0,88	1,21	17,2	38,3%
			3	1236	43,7	1440	186		0,88	1,17	17,0	36,5%