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THERMACULTURE ON 'CABERNET SAUVIGNON' VINEYARD INCREASES WINE PIGMENTS AND WINE SENSORY QUALITY

THERMACULTURE EM VINHEDOS DE 'CABERNET SAUVIGNON' AUMENTA OS PIGMENTOS DO VINHO E A QUALIDADE SENSORIAL DO VINHO

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SUMMARY

Thermaculture is applied in vineyards for fungus diseases prevention. However, it can also induce plant to thermal stress, modulating the secondary metabolism. Thus, it was determined the role of thermaculture on 'Cabernet sauvignon' wine sensory properties and anthocyanin profile by the application on the vineyard. A total of 19 applications from flowering to harvesting were made. After winemaking and aging (12 months), physicochemical analysis, anthocyanin characterization by high performance liquid chromatographic (HPLC-PDA-MS/MS), and sensory descriptive analysis were done. Treatment on grapes has increased 4.5% of total anthocyanins monomers, 29.4% of pyranoanthocyanins, and 29.5% of direct condensation products that were formed in wine. Thermaculture affected the sensory quality of wine since judges have perceived significant differences in visual (tear, red color) and gustatory (woody flavor) attributes. Finally, thermaculture is an innovative technology and a promising tool to increase anthocyanins in 'Cabernet Sauvignon' wines.

RESUMO

Thermaculture é aplicada em vinhedos para prevenção das doenças fúngicas. Contudo, pode induzir a planta a um estresse térmico, modulando o metabolismo secundário. Assim, foi determinado o papel da *thermaculture* nas propriedades sensoriais e perfil das antocianinas do vinho 'Cabernet Sauvignon' pela aplicação na vinha. Foi realizado um total de 19 aplicações do florescimento à colheita. Após a vinificação e conservação (12 meses) foram realizadas análises físico químicas, caracterização das antocianinas por cromatografia líquida de alta eficiência (CLAE – DAD – EM/ EM), e análise descritiva quantitativa. O tratamento nas uvas aumentou 4,5% dos monômeros de antocianinas totais, 29,4% das piranoantocianinas e 29,5% nos produtos de condensação direta que se formaram no vinho. A *thermaculture* afetou a qualidade sensorial do vinho, uma vez que os julgadores perceberam diferenças significativas nos atributos visuais (lágrima, cor vermelha) e gustativos (sabor amadeirado). Finalmente, a *thermaculture* é uma tecnologia inovadora e uma ferramenta promissora para aumentar as antocianinas nos vinhos 'Cabernet Sauvignon'.

Key words: treatment thermal, *Vitis vinifera*, malvidin-3-glucoside, quantitative descriptive analysis.
Palavras-chave: tratamento térmico, *Vitis vinifera*, malvidina-3-glucosídeo, análise descritiva quantitativa.

INTRODUCTION

Brazilian viticulture is a growing national agro-industrial activity with a grape annual production of more than 70.0 million kilograms only for *Vitis vinifera* L. (Uvibra, 2015). Nowadays, new territorial spaces are being tested to increase yield and grape quality. One of these areas is a plain grassland territory located in the east of the parallel 30°S in which *Vitis vinifera* grapes for winemaking are produced. *Vitis vinifera* cultivars were adapted to Brazilian climate having a median production with

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two distinct annual growing cycles (hibernal rest and crop growing) (Protas *et al*., 2014). Region climate is characterized by high temperatures in the summer and a cold and humid winter, with high rain indices (INMET, 2016). Wet climates and high rain incidence (Bellí *et al*., 2005) are some of the reasons by which fungus contamination is developed in vineyards (Wilcox, 2016) compromising grape integrity and hygienic safety.

Thermaculture or Thermal Pest Control (TPC) technology was implemented in some vineyards to help the control of fungus diseases. A previous preliminary study using temperatures of 110 ºC in air by a TPC equipment has shown that it was able to control fungus diseases and decrease insects (Barra, 2006). Furthermore, there are some evidences that this technology can also act on plant secondary metabolism in answer to a thermal stress, acting as an abiotic elicitor (Fischer, 2012). Phenolic compounds, as anthocyanins, are secondary metabolites that have their synthesis stimulated during plant stressing situations (Jochum *et al*., 2007; Treutter, 2010). Therefore, they could be found at increased levels in fruits previously treated by thermal treatment during crop management.

Anthocyanins are major pigments in grape and grape products. These compounds could have increased levels due to many environmental stressors, including increased solar UV radiation and temperature (Ramakrishna and Ravishankar, 2011). In literature, wine pigments are formed during vinification and aging (pyranoanthocyanins, direct condensation products, and acetaldehyde mediate products) and they are linked to some important sensory properties of wine, such as color (He *et al*., 2012a,b) and some mouthfeel perceptions (Vidal *et al*., 2004).

Determination of the anthocyanin profile in 'Cabernet Sauvignon' wines using appropriate and high selective and sensible techniques, as high performance liquid chromatographic (HPLC-PDA-MS/MS) are vastly reported in literature (Alañón *et al*., 2016; Avizcuri *et al*., 2016). Nonetheless, no previous studies about the effect of thermaculture practices on wine anthocyanin profile and sensory properties were found.

Thus, this work aimed to determine the effect of thermaculture on the wine anthocyanins and quality by the application of the TPC technique in 'Cabernet Sauvignon' grapes. It is not proposed to control pests with TPC and how it is successful or not. Indeed, the manuscript deals with a new application of TPC and not to the verification of the equipment effectiveness to the purpose by which it was delineated by manufacturers.

MATERIAL AND METHODS

Experimental design for TPC treatment

This experiment was conducted in two consecutive years (2013/2014). The vineyard of the red cultivar 'Cabernet Sauvignon' (*Vitis vinifera* L.) used in the experiment was located in Estância Leões (30°59′9.789″S, 54°29′40.751″W, elevation 260 m). It was planted in 2003, grafted on SO4 rootstock, and located at the city of Dom Pedrito (Rio Grande do Sul, Brazil). Vines were trained to a vertical trellis on a single cordon system. Spacing was 3.3 m between rows and 1.00 m between plants. Rows were North-South oriented. The soil is classified as typical Ortic Haplic Luvisol as described by Streck *et al*. (2008) using the soil classification of Embrapa (2006). Climate is characterized as humid subtropical climate (Cfa) by the Köppen classification.

One block from vineyard was split in two different zones for control and TPC treatment with enough separation. Thus, experimental zones for control and TPC consisted of six lines, which were separated from each other by three lines that were not used in the experiment (border, a total of 6 m from the line beginning). In each line, one grape cluster was collected every 10 m from the border (6 m from the line beginning). All samples were collected at 1.3 m from the soil. A detailed experimental design map developed by Cecília Kaehler Sautter is provided (Figure 1) for the illustration of the adopted field treatment. Total harvested grape amount (15 kg for each experimental group, control and TPC) was homogenized and randomly divided in four replications (~3.8 Kg each).

TPC equipment (Lazo TPC, model GLP granel) was towed by a tractor (John Deere, model 5603) which moves at a speed of 4 km/h releasing the heated air (130 ºC) at a distance of 20 cm from espaliers. A total of 19 applications were made from flowering to harvesting. At the commercial maturity degree, grapes were harvested (Control – Total soluble solids (TSS): 20.2 ºBrix, pH: 3.94, total acidity (TA): 0.56 g/100 mL; TPC - TSS: 20.2 ºBrix, pH: 3.98, TA: 0.56 g/100 mL).

Wine making practices

From each field replication one wine making process was performed in a total of eight batches. A micro scale wine making processing technology was made as preconized by Pszczólkowski and Lecco (2011) with some modifications.

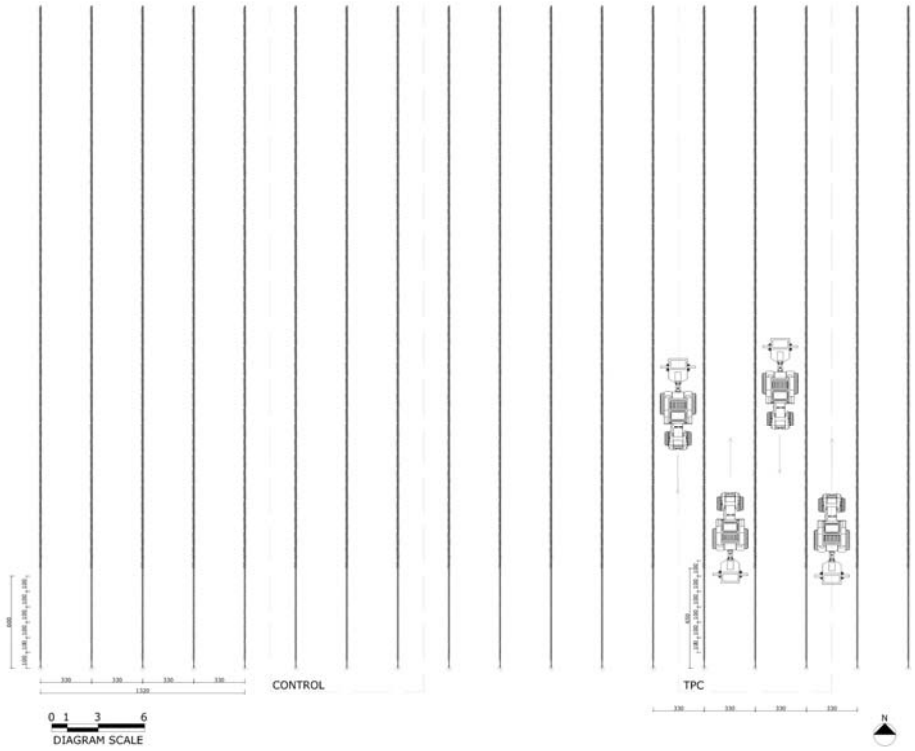

Figure 1. Experimental design of the vineyard with control and TPC treatments.

Desenho experimental do vinhedo com tratamento controle e TPC.

Must was placed in micro fermenters (2 L) and added of 50 mg/L sulphite, pectolytic enzymes (5g of each enzyme preparation/100 Kg of must, Lafase® He Grand Cru and Lafase® fruit, Laffort® Company, Bordeaux, France), yeast (20g yeast preparation/hL, Zymaflore® FX10, Laffort®), and probiotic additives (30g additive preparation/hL Superstart® and 40g additive preparation/hL Bioactiv®, respectively, Laffort®). Temperature of fermentation was kept at 25 °C and cap management was performed three times per day by closed fermenter stirring.

Malolactic fermentation was controlled by paper chromatography according to Daudt (1971). After malolactic fermentation, wines were filtered, bottled and stored for 12 months.

Identification by HPLC-PDA-MS/MS and quantification by HPLC-PDA analysis

Dealcoholized wine samples (5 mL, 39 °C for 10 min; Rotavapor® R-300, Buchi, Labortechnik, Germany) were purified in solid phase extraction cartridge (SPE-C18) using the method described by Rodriguez-Saona and Wrolstad (2001) for identification. For quantification, dealcoholized wine samples were only filtered (0.22 µm, PTFE, Phenomenex) before injection.

Identification was performed in HPLC-PDA-MS^n connected to ion trap mass spectrometer (Esquire 6000, Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray interface operating in positive mode. The ESI conditions were as follows: capillary voltage of +4.5kV, nebulizer gas pressure at 30 psi, dry gas at 11 mL/min, and gas temperature at 310 °C. MRM experiments were performed in a full scan range of 200 to 1000 m/z of all fragments formed from 3 major parent ions per second. Quantification was performed in HPLC-PDA equipment was a Prominence 20 A (Shimadzu, Japan) equipped with degasser (DGU20A5 prominence, Shimadzu, Japan), column oven (CTO-20A prominence, Shimadzu, Japan) and coupled to a PDA detector (SPDM-20A prominence, Shimadzu, Japan).

Identification and quantification were performed using a reverse phase column (C18, 2.6 µm, 100 mm x 4.6 mm, Phenomenex, Torrance, USA)

thermostated at 38˚C and gradient elution for separation was performed using two mobile phases: (A) acidic solution (3% v/v of formic acid, 85%, Merck®) in ultrapure water (Milli-Q Gradient System, Millipore Corporation, Massachusetts, EUA); (B) pure acetonitrile (HPLC grade, Merck, Darmstadt, Germany). Thus, elution gradient has as initial condition 10% B and 90% A. During the first 20 minutes, gradient has increased to 25% of B. After 20 minutes, gradient was increased to 80% of B in 5 minutes, kept in this condition for 2 minutes to elute highly retained compounds from injected 'Cabernet sauvignon' wines. After column cleanup, the initial conditions were re-established kept for 5 minutes. All eluted compounds were monitored from 250-800 nm. A standard curve of malvidin-3-glucoside (Oenin chloride, 97%, Sigma-Aldrich, St. Louis, MO, U.S.A) was developed and analyzed in relation to linearity, limits of detection (LoD) and quantification (LoQ) as recommended by the International Chemical Harmonization guide (ICH, 2005).

Physicochemical and color analysis

Wines were analyzed for alcohol concentration using a digital distilling unit (Gibertini, model VAD33) and total acidity (TA) according to OIV (2011). Procyanidins (PROC), color intensity (CI), and all other color parameters were determined according to Ribèreau-Gayon *et al*. (2006). Monomeric anthocyanins (MA) were determined using the method described by Giusti and Wrolstad (2005) and total polyphenols (TP) by Singleton and Rossi (1965).

Quantitative Descriptive Analysis (QDA)

'Cabernet sauvignon' wines were analyzed using QDA procedures as described by Stone and Sidel (2004) with modifications. All sessions were performed in the laboratory facilities for sensory analyses from the Food Science and Technology department of UFSM (Universidade Federal de Santa Maria) after approval by the ethical committee (protocol# 23044813.1.0000.5346). The panelists were selected and trained as described by Behrens and Silva (2000) using tests for basic taste recognition by triangular discriminative analysis and specific sensory training sessions. Descriptors for aroma were determined as described in the "Standard Terminology of Wine Aroma" published by Noble *et al*. (1987). Panelists must have 90% of accuracy in three test repetitions to be selected as trained. The final trained panel (n=8, 7 women, 1 man, 24-29 years old) have established descriptors and standards that should be used to describe 'Cabernet sauvignon' wines in an additional sensory session (Table I). All sensory sessions were performed in appropriate closed cabins with white tables equipped with openable windows for samples delivery. Sessions were a total of two hours long per day, and they were performed twice a week, adding up to a total of six sessions (three weeks). In each session, a total of four samples from different experimental groups were analyzed. All attributes were analyzed using a non-structured scale of 9 cm that started with the word "nothing" and "weak" as the minimal score, and ended with the word "strong" for the maximum score.

Statistical treatment of data

All analyses were done in triplicate for each experimental repetition (n=4). Data was submitted to one-way analysis of variance (ANOVA) and mean comparison was performed by Tukey test at 5% of error probability. The software Statistica® 9.0 was used for statistical treatment of data.

RESULTS AND DISCUSSION

Physicochemical and color analysis

All physicochemical wine quality parameters were in agreement with local and international regulation (Brasil, 1988; Official Journal of the European Union, 2006). Furthermore, except for alcohol concentration (Table II), results from physicochemical analyses were not different ($p \leq 0.05$) between 'Cabernet sauvignon' wines produced from control and from TPC treated samples. Alcohol concentration of wines from TPC treated grapes was significantly lower than that of control samples; however, it is still in agreement with the Brazilian regulation (Brasil, 1988) and international laws (Official Journal of the European Union, 2006) for red wine.

Wine color analysis (Table II) has shown a treatment effect in parameters related to the overall color intensity (CI), hue angle, and the absorbance at 520 nm which is highly related to the anthocyanins levels and their related pigments composition. However, no significant differences were observed in the total levels of monomeric anthocyanins. It may indicate that colorimetric method is not enough sensitive to detect oscillation on these compounds that could justify differences in color. Moreover, the difference ($p \leq 0.05$) in hue values due to TPC treatment possibly indicates that anthocyanin composition could also be affected. Thus, a high selective and sensitive analytical method by HPLC-PDA-MS/MS was required and used to determine the treatment effects in each anthocyanin and in the pigment profile as a whole. Moreover, it is interesting to highlight that total monomeric anthocyanins by pH-differential method in young wine (6 months) were also determined. This result clearly shows that TPC young wine had 20% more monomers of anthocyanins than

the control (Control: 145.1 mg/L and TPC: 179.9 mg/L). It is evident that thermaculture induced an increase of anthocyanins in grapes and it may affect the wine quality. Since commercially available wines usually have one year of aging to acquire proper sensory quality, it was decided to perform sensory analysis, HPLC quantification, and LC-MS/MS identification only in one-year aged wine samples.

Table I

Sensorial descriptors, definitions and references used in the evaluation of samples of 'Cabernet Sauvignon' wine

Descritores sensoriais, definições e referências utilizados na avaliação das amostras de vinho 'Cabernet Sauvignon'

Descriptor	Definition	Reference
	VISUAL	
Red rubi Color	High Rubi red color is characteristic of young wines.	Weak: 50 mL of 'Cabernet Sauvignon' wine in 50 mL of water Strong: 100 mL of 'Cabernet Sauvignon' wine in 15 drops of dye
Viscosity	It is perceived by wine tears in glass.	Little: 10 mL of brandy (40%) in 90 mL water Very: 100 mL of brandy (40%)
Translucent	Light scattering by the wine in the glass.	Little: 50 mL of grape juice in 50 mL of water Very: 100 mL of grape juice
	OLFATORY	
Woody	Aroma that reminds wood, tobacco, and toast.	Nothing: 10 mL of 'Cabernet Sauvignon' wine Strong: 10 mL of reserve 'Cabernet Sauvignon' wine/One cigarette tobacco
Herbaceous	Aroma that reminds herbs and plants.	Nothing: 10 mL of water Strong: 10 g of sliced fresh pepper/10g of olive
Alcohol	Aroma perception of ethanol.	Weak: 10 mL of 5% v/v ethanol in water Strong: 10 mL of 20% v/v ethanol in water
Red fruits	Aroma of berries and small red fruits.	Nothing: 10 mL of water Strong: 10g of plum/strawberry/raspberry/blueberry
Ripe fruits	Aroma from mature and dry fruits.	Nothing: 10 mL of water Strong: 10 g of raisin/ 10g of ripe plum/
Caramelized	Aroma from caramel and vanilla.	Nothing: 10 mL of water Strong: 3 drops of 0.25% of vanillin solution/ 10g of brown sugar
Floral	Aroma from flowers mainly rose and violets.	Nothing: 10 mL of water Strong: 10 mL of water with 1 drop of rose aroma
	GUSTATORY	
Fruit	A taste that is associated to berry and small red fruits.	Nothing: 10 mL of 'Cabernet Sauvignon' wine Strong: 10 mL of 'Cabernet Sauvignon' wine with 10 g of plum/strawberry/blueberry
Woody	Wood, tobacco, coffee, and toast taste perceptions.	Nothing: 10 mL of 'Cabernet Sauvignon' wine Strong: 10 mL of reserve 'Cabernet Sauvignon' wine
Acid	Sour taste perceived on lateral parts of the tongue.	Weak: 10 mL of wine with 0.02% w/v of citric acid Strong: 10 mL of wine with 0.9% w/v of citric acid
Bitter	Mainly perceived on the back part of the tongue reminding coffee bitterness.	Weak: 10 mL of wine with 0.01% w/v of caffeine Strong: 10 mL of wine with 0.1% w/v of caffeine
Astringent	Dry and roughness mouth perception	Weak: 10mL of wine Strong: 10 mL of wine with the addition of 1 ml of grape seed infusion
Pungent	Spicy and acrid taste perception.	Weak:10 mL of wine Strong: 10 mL of wine with 2 sliced ginger
Persistence and Body	Aftertaste perception immediately after swallow. The mouthfeel perception of fullness.	Weak:10 mL of 'Cabernet Sauvignon' wine Strong: 10 mL of reserve 'Cabernet Sauvignon' wine

Table II

Physicochemical behavior and color of wines produced from grapes treated with TPC*

*Comportamento físico químico e cor dos vinhos produzidos a partir de uvas tratadas com TPC **

Parameters		Experiment	
		Control	TPC
Physiochemical measurements	Alcohol	11.8±0.23 a	11.4±0.00 b
	pH	3.58±0.00 a	3.66±0.10 a
	TA	107.5±2.9 a	110.0±11.5 a
Color measurements	A_{420nm}	0.19±0.01 a	0.20±0.00 a
	A_{520nm}	0.19±0.00 b	0.24±0.00 a
	A_{620nm}	0.03±0.00 a	0.04±0.00 a
	CI	0.42±0.01 b	0.49±0.00 a
	Hue	1.03±0.09 a	0.85±0.00 b
Phytochemical total measurements	PROC	1.32±0.06 a	1.40±0.03 a
	MA	135.02±16.9 a	144.60±3.11 a
	TP	1353.2±8.07 a	1359.4±25.22 a

*Means followed by different letters in a row are statistically different ($p<0.05$); TA - total acidity (meq/L), PROC - procyanidin (g/L), A - absorbance, CI - color intensity (absorbance), MA - monomeric anthocyanin (mg/L), TP - total polyphenol (mg/L).

Results from total polyphenolic assay and proanthocyanidin quantification in wines were not affected by TPC in grapes.

Identification and quantification of 'Cabernet sauvignon' wine pigments

A total of 24 different peaks were detected by the HPLC-PDA method in which the combined information of elution order, ultraviolet-visible (UV-Vis) spectra, and mass spectrometry fragmentation patterns allowed the identification of more than 30 different anthocyanins and related pigments in 'Cabernet sauvignon' wines (Figure 2, Table III). No new compounds in wines were detected as result of TPC treatment on grapes. However, concentration of some of the identified pigments were increased due treatment. Identification of each of these peaks will be discussed in separate followed by a comparison of data from quantification assay of wines produced with treated and untreated samples. HPLC-PDA method used for quantification was linear ($r^2 = 0.991$) in the studied range (0.5-60 mg/L) with no lack-of-fitness. Lower limits of quantification (0.33 mg/L) and detection (0.10 mg/L) allowed the analysis of all peaks at 520 nm.

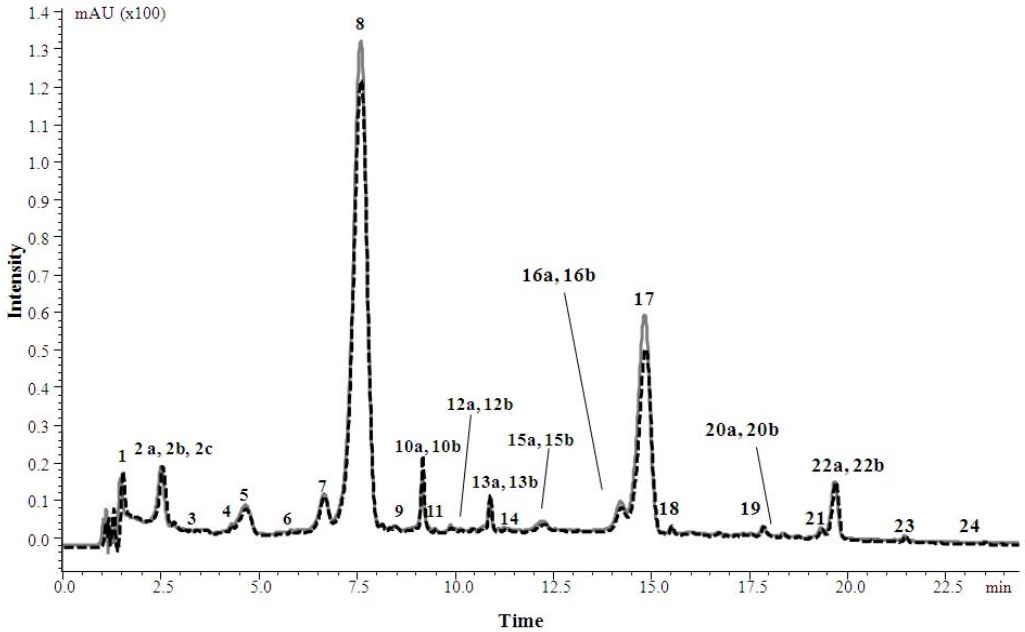

Figure 2. Chromatographic separation of anthocyanins and related red wine pigments detected in 'Cabernet Sauvignon' wine samples. Continuous line corresponds to the wine obtained from grapes that have received TPC in the vineyard. Dashed line is from wine produced with control grapes.

Separação cromatográfica das antocianinas e pigmentos vermelhos do vinho detectados nas amostras de vinho 'Cabernet Sauvignon'. A linha contínua corresponde ao vinho obtido a partir de uvas que receberam TPC *no vinhedo. A linha tracejada é correspondente ao vinho produzido com uvas controle.*

Table III

Tentative identification and quantification of individual anthocyanins and derived anthocyanins pigments in 'Cabernet Sauvignon' wines subjected to TPC[a]

Tentativa de identificação e quantificação individual das antocianinas e pigmentos derivados de antocianinas em vinhos 'Cabernet Sauvignon' submetidos ao tratamento TPC[a]

Peak	RT[a] (min)	M^+	MS^2 (Product ions)	λ_{max} (nm)	Pigment family	Compound	Experiment	
							Control	TPC
1	1.45	797	635, 467	527	Direct condensation product	Mv-3-hex-GC	2.01±0.10 b	2.72±0.06 a
2a	2.56	751	619	524	Direct condensation product	Pn-3-hex-C		
2b	2.57	465	303	524	Anthocyanin	Dp-3-hex	2.76±0.57 a	2.88±0.07 a
2c	2.58	781	619, 373	524	Direct condensation product	Mv-3-hex-C		
3	3.63	533	371	517	Pyranoanthocyanin	A type vitisin of Dp-3-hex	0.19±0.02 b	0.22±0.00 a
4	4.20	573	371	527	Pyranoanthocyanin	A type vitisin of Dp-3-acetylhex	0.17±0.04 a	0.24±0.00 a
5	4.74	479	317	525	Anthocyanin	Pt-3-hex	2.56±0.25 a	2.70±0.03 a
6	5.70	655	331	527	Anthocyanin	Mv-3,5-dihex	0.02±0.02 a	0.05±0.00 a
7	6.70	463	301	516	Anthocyanin	Pn-3-hex	1.98±0.11 b	2.90±0.01 a
8	7.66	493	331	525	Anthocyanin	Mv-3-hex	56.8±12.9 a	61.7±0.14 a
9	8.96	823	619, 467, 373	527	Direct condensation product	Mv-3-acetylhex-C	0.19±0.06 a	0.13±0.00 a
10a	9.16	507	303	510	Anthocyanin	Dp-3-acetylhex	1.75±0.33 b	2.50±0.09 a
10b	9.17	561	399	510	Pyranoanthocyanin	A type vitisin of Mv-3-hex		
11	9.34	547	385	485	Pyranoanthocyanin	A-type vitisin of Pt-3-hex	0.04±0.01 b	0.08±0.00 a
12a	9.86	517	355	488	Pyranoanthocyanin	B type vitisin of Mv-3-hex	0.19±0.02 a	0.19±0.01 a
12b	9.87	943	635	488	Direct condensation product	Adduct gallocatechin-mv-3-O-hex		
13a	10.85	801	331	514	Direct condensation product	Mv-3-trans-cmhex5-hex	0.93±0.17 b	1.31±0.00 a
13b	10.86	603	399	514	Pyranoanthocyanin	A type vitisin of Mv-3-acetylhex		
14	11.23	491	287	527	Anthocyanin	Cy-3-acetylhex	0.08±0.00 a	0.08±0.00 a
15a	12.21	521	317	527	Anthocyanin	Pt-3-acetylhex	0.81±0.10 a	0.61±0.00 b
15b	12.22	559	355	527	Pyranoanthocyanin	B type vitisin of Mv-3-acetylhexoside		
16a	14.17	505	301	528	Anthocyanin	Pn-3-acetylhex	1.94±0.31 a	1.98±0.00 a
16b	14.18	927	619, 467	528	Direct condensation product	Mv-3-(p-coumaroyl)hex-C		
17	14.79	535	331	527	Anthocyanin	Mv-3-acetylhex	24.0±5.02 a	22.0±0.04 a
18	15.43	707	399	515	Pyranoanthocyanin	A type vitisin of Mv-3-(p-coumaroyl)hex	0.19±0.02 b	0.29±0.00 a
19	17.76	639	331	530	Anthocyanin	Mv-3-cis-(p-coumaroyl)hex	0.40±0.00 b	0.42±0.00 a
20a	18.25	847	685	458	Other compounds	Unknown	0.10±0.00 b	0.14±0.00 a
20b	18.26	817	655	458	Other compounds	Unknown		
21	19.22	609	301	520	Anthocyanin	Pn-3-(p-coumaroyl)hex (cis)	0.55±0.01 a	0.45±0.00 b
22a	19.56	639	331	527	Anthocyanin	Mv-3-trans-(p-coumaroyl)hex	4.50±0.77 a	3.76±0.02 a
22b	19.57	625	463	527	Pyranoanthocyanin	Mv-3-hex-4-vinylcatecol		
23	21.32	609	447	503	Pyranoanthocyanin	Mv-3-hex-4-vinylphenol	0.21±0.03 a	0.23±0.00 a
24	23.33	651	447	505	Pyranoanthocyanin	Mv-3-acetylhex-4-vinylphenol	0.05±0.01 a	0.04±0.00 a

[a]RT: retention time (min); M+: positive charged molecular íon; MS^2: fragmentation of M+; Dp-Delphinidin, Cy-Cyanidin, Pt-Petunidin, Pn-Peonidin, Mv-Malvidin, C-Catechin, GC-Gallocatechin, hex-Hexoside. Means followed by different letters in a row are statistically different ($p<0.05$). Concentration expressed as malvidin-3-glucoside (mg/L).

Anthocyanins monomers

'Cabernet Sauvignon' wines have shown 13 different monomers of anthocyanin (peaks 2b, 5-8, 10a, 14, 15a, 16a, 17, 19, 21 and 22a, Table III, Figure 2). In this class of anthocyanin, only one compound was a dihexoside anthocyanin (Malvidin-3,5-dihexoside, 5.70 minutes) identified by two neutral losses of 162 m/z and with a shorter elution time than the monohexoside form (Malvidin-3-hexoside, 7.66 minutes). The natural occurrence of hexoses linked to the main rings of anthocyanins in 'Cabernet sauvignon' grapes was mostly identified as glucose (De la Cruz *et al*., 2012). The presence of only one anthocyanin with two hexose moieties in smaller concentration than all other compounds (0.02 mg - control and 0.05 mg - TPC of Mv-3-hex equivalents/L wine) is in agreement with the literature, which recognizes wines from *Vitis vinifera* species as having small quantity of diglucosides (Lambert *et al*., 2011; Manns and Mansfield, 2012). It was also reported in 'Cabernet sauvignon' wines (He *et al*., 2012b; Pedastsaar *et al*., 2014). Moreover, TPC has not significantly altered the concentration of this compound.

Four anthocyanins linked to hexose were found in samples of 'Cabernet sauvignon' wines (peaks 2b, 5, 7 and 8, Table III, Figure 2) identified by a single neutral loss of 162 m/z from the molecular ion and detection of the aglycon ion (303, 317, 301, and 331 for delphinidin, petunidin, peonidin, and malvidin, respectively). Elution order was determined by substitution pattern in the aglycon with shorter times for high hidroxylated (delphinidin) than for methoxylated (malvidin) compounds. Only peonidin-3-hexoside had its levels increased in wine by TPC treatment in the vineyard (46.46% higher in TPC treated samples than in control).

Accordingly to the expected sequence in the elution order for anthocyanin, in 'Cabernet sauvignon' wines five acetyl derivate compounds (peaks 10a, 14, 15a, 16a, and 17, Table III, Figure 2) were detected with longer retention times than non-acylated compounds and with the characteristic fragmentation pattern in mass spectrometry. *p*-coumaroyl derivatives were also found in these samples linked to peonidin and malvidin; this last anthocyanin was in two isomeric forms determined by same fragmentation patters but in two different retention times. In literature, it is already established that *cis* isomers have a lesser intense retention in C-18 columns than *trans* structures (Alcalde-Eon *et al*., 2006; Boido *et al*., 2006). In the present study, *p*-coumaroyl derivatives were less abundant than acetyl derivatives. This observed profile behavior is in agreement with previous literature data for 'Cabernet sauvignon' wines (Arozarena *et al*., 2002; Burns *et al*., 2002).

TPC caused a significant increase of only two of the *p*-coumaroyl derivatives, the malvidin-3-*cis*-(*p*-coumaroyl)hexoside and the peonidin-3-(*p*-coumaroyl)hexoside. For acetyl derivatives, treatment could have affected delphinin-3-acetylhexoside and petunidin-3-acetylhexoside. However, it cannot be affirmed because in these PDA-detected peaks (10a/10b and 15a/15b) there are co-elutions with pyranoanthocyanins. For peak 10a/10b (delphinidin-3-acetylhexoside plus A type visitin of Mv-3-hexoside), it could be assumed that peak is majorly composed of the pyranoanthocyanin due to the characteristic UV-visible spectra with the maximum absorbance at 510 nm, a 15 nm hypsochromic shift from the precursor anthocyanin (malvidin-3-hexoside, 525 nm).

For this class of anthocyanins pigments, which derived from grape, all the compounds detected in 'Cabernet sauvignon' wines were previously reported for different wines obtained from the same grape variety (Arozarena *et al*., 2002; Burns *et al*., 2002; He *et al*., 2012a; Pedastsaar *et al*., 2014).

Pyranoanthocyanins

Eleven pyranoanthocyanins were detected in wine samples (peaks 3, 4, 10b, 11, 12a, 13b, 15b, 18, 22b, 23, and 24) in which only two of them were formed by cycloaddition acetaldehyde into anthocyanin structure (B type vitisins). Identification of these compounds was based on the detection of characteristic fragments with m/z values that were equal to the mass to charge ratio of the aglycon plus 68 amu and 24 amu for A type and B type vitisins, respectively. Among all these compounds, at least three of them seem to be altered by TPC treatment in the vineyard since they were detected in higher amounts and as pure compounds in peaks with no co-elutions. These anthocyanin derived pigments are formed due cycloaddition of fermentation products (pyruvic acid and acetaldehyde) during winemaking and aging, and they were already reported in the literature for 'Cabernet sauvignon' wines (Aguirre *et al*., 2011; Alañón *et al*., 2016).

Direct condensation products

Wine samples are recognized as having great amount of other polyphenols in addition to anthocyanins. Thus, by electrophilic attack, flavanols such as catechin and gallocatechin can easily form adducts with anthocyanins. These compounds were already detected in a different range of wines samples (Alcalde-Eon *et al*., 2006; Gordillo *et al*., 2012). In the present work, a total of seven direct condensation products were identified in 'Cabernet sauvignon' wines in both experimental groups. However, only one of them (peak 1, Table III) was found in

significantly higher concentration in wine produced with grapes from a TPC treated vineyard.
TPC has increased 4.5% the total content of anthocyanins monomers, 29.4% the pyranoanthocyanins and 29.5% the direct condensation products. The increase in anthocyanins monomers was not detected by the colorimetric measurement by pH differential method (Table II) possibly by the smaller sensitivity of this method in comparison with HPLC-PDA-MS/MS techniques. Moreover, the greatest increases in pigments were in derived-anthocyanin pigments (pyranoanthocyanins and direct condensation products), which are not properly measured by this colorimetric assay. The anthocyanin increments due to TPC detected by HPLC is in agreement with color results (Table II), which were significantly affected in intensity and in absorbance at 520 nm.
The TPC wine presented greater score values for visual tear and gustatory woody perception than the wine produced with untreated grapes ($p \leq 0.05$, Figure 3).

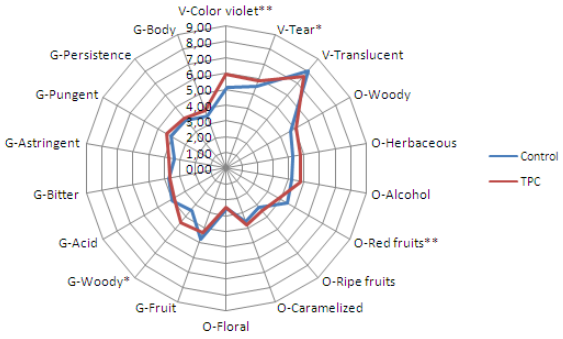

Figure 3. Effect of TPC in 'Cabernet Sauvignon' wines. V – visual; O – olfatory; G – gustatory. Significance level of the ANOVA (*$p \leq 0.05$; **$p \leq 0.10$).

Efeito do TPC em vinhos 'Cabernet Sauvignon'. V – visual; O – olfatório; G – gustatório. Nível de significância da ANOVA ($p \leq 0.05$; **$p \leq 0.10$).*

Sensory analysis

Visual tear is related to wine viscosity and, thus, any substance that could alter this property will also affect this sensory parameter. Alcohol content (Table II) in 'Cabernet sauvignon' wines produced with TPC treated grapes was lower than in the wine manufactured with grapes from control groups. The relationship between wine tear intensity and its ethanol content is still unclear in literature. Moreover, other compounds, such as condensed tannins, can also change wine viscosity altering its sensory perception. This class of polyphenols had showed a tendency to increase due TPC as determined by colorimetric measurements (p-value of 0.11, Table I). Another evidence that could support the hypothesis that TPC is altering tannin content and composition is the increased levels of direct condensation products of anthocyanins with proanthocynidin monomers (catechin and epicatechin) in wines from TPC group (29.5%).

During winemaking and aging no wood materials or barrels were used. However, the woody gustatory descriptor was detected in all samples, inclusive, with higher scores in wines produced with TPC treated samples ($p \leq 0.05$). Previous literature has already established that wood flavor is a combination of wood, toasted coffee, tobacco, and phenolic compounds (Arenhart, 2015). Phenolic compounds were also linked to aroma of wood in wines (Noble *et al*., 1987). Since control samples have also this flavor, a polyphenol could have some contribution to the overall perception of wood for the grape variety used in this work. Moreover, TPC grape treatment had significantly affected wine pigments that are mostly formed during aging (pyranoanthocyanins and direct condensations products). These compounds could also be enrolled in the woody taste by the increase in phenolic gustative perception. Previous literature has already reported that anthocyanins could have some relationship with gustatory sensory wine descriptors, such as wine mouth fullness (Vidal *et al*., 2004).

At 90% of confidence level, 'Cabernet Sauvignon' wines were affected in sensory descriptor for olfactory red fruits aroma and visual red color intensity ($p \leq 0.10$, Figure 3). Increased perception of red color in 'Cabernet Sauvignon' wines by TPC grape treatment is in accordance with results obtained by instrumental analysis for color characterization (Table II) which has shown greater absorbance at 520 nm and color intensity ($p \leq 0.05$). Moreover, it is acceptable that an instrumental analysis should be more sensitive than an experiment with human perception for discrimination. Individual anthocyanins and wine anthocyanin derived pigments (Table III, Figure 2) was also increased in wines from treated samples, which also corroborates the greatest perception of visual red wine.
Red fruits aroma was already observed in 'Cabernet Sauvignon' wines (Chapman *et al*., 2004; Bindon *et al*., 2014) and it was also perceived in our sensory tests. However, treatment was not able to decrease this sensory characteristic ($p \leq 0.05$), and it has just shown a tendency to reduce it ($p \leq 0.10$).

CONCLUSIONS

Thermal treatment of 'Cabernet sauvignon' grapes in the vineyard has increased the concentration of anthocyanins and, in a greater extension, the derived pigments formed during winemaking and aging. The results suggested that TPC could stimulate secondary metabolism increasing levels of these polyphenols. It should be highlighted that significant differences in anthocyanins were just perceived in HPLC analysis and not by colorimetric measurement. Thus, other classes (proanthocyanidins and other non-anthocyanin polyphenols) that do not differed by simple measurements (colorimetric/spectrophotometric assays) could be better tracked if highly selective and sensible techniques are applied, as HPLC and mass spectrometry.

TPC has significantly affected the sensory quality of wine, especially the visual attributes (perception of tear, red color) and gustatory perceptions (woody flavor).

Thermaculture is an innovative technology for crop research and it could be a promising tool to increase anthocyanins and wine quality of 'Cabernet Sauvignon'. Future researches with the same specie could be performed to determine how TPC conditions should be adjusted in different regions and crop years and determine soil and climate interactions with TPC effect. Moreover, HPLC-PDA-MS/MS techniques should be used to determine with greater sensitivity the effect of TPC in tannins and other polyphenolic classes that are also relevant for wine quality.

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