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Evolution of Polyphenols, Volatile Aroma Compounds and Natural Yeast Flora of Coda di Volpe White Grape

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Wine is one of the main product of the agro-food products in Italy especially for its economic importance to local and regional level. The Campania region is excellent for the production of wine especially those of value, thanks to the presence of wide known varieties of native grapes. The aim of this work is been to characterize the chemical, biochemical and microbiological properties of Coda di Volpe grapes, cultivated in two areas, the hills and flat lands of Benevento. The name Coda di Volpe means tail of the fox, which was given in reference to the varieties of long, pendulous bunches of grapes, which resembles a fox's bushy tail.
The polyphenols, pH, total acidity and °Brix were determined at different stages of maturation in order to identify the optimal time for the harvest. In particular, the total polyphenols content was evaluated with Folin-Ciocalteu method, while the specific polyphenols were determined by RP-HPLC. The grapes were harvested in sterile bag, sealed, pressed and incubated at 20°C for 1 month. During the fermentation it was isolated epiphytic microflora and characterized in order to analyse the physiological, oenological and technological properties. Three different yeast strains were assayed in their oenolegical ability, these were used in experimental fermentation with Coda di Volpe must. During alcholic fermentation have been evaluated yeast population, alcoholic grade and the volatile profile by the SPME-GC/MS technique. The data obtained have highlighted their ability to conduct the fermentation process, dominate the indigenous microflora and contribute to the definition of the flavour profile.

1. Introduction

Coda di Volpe is a golden-yellow grapes variety, used to produce wines in and around the region of Campania in southern Italy. The wine produced from this cultivar is often described as medium to medium-full bodied, fruity (citrus, peach, pineapple) and spicy (nutmeg, cinnamon) and flavors of grapefruit, lemon, and almond.
Since the 1980's, wineries in Campania have begun making single-varietal wines from Coda di Volpe, and now used in the DOC wines of Irpinia and Sannio area.
Industrial wine fermentations are currently conducted by starters of special wine yeast strains of *Saccharomyces cerevisiae* in contrast to traditional spontaneous fermentations conducted by the flora present on the grapes and in the winery. Despite the advantages of using pure cultures of *S. cerevisiae* with regard to the easy control and homogeneity of fermentations, wine produced with pure yeast monocultures lacks the complexity of flavour, stylistic distinction and vintage variability caused by indigenous yeasts (Swiegers et al., 2005). In recent years the inclusion of non-*Saccharomyces* wine yeast species as part of mixed starters together with *S. cerevisiae* to improve wine quality has been suggested as a way of taking advantage of spontaneous fermentations without running the risks of stuck fermentations or wine spoilage (Romano et al., 2003; Ciani et al., 2006). Although non-*Saccharomyces* wine yeast species have traditionally been associated with high volatile acidity, ethyl acetate production, off-flavours and wine spoilage (Sponholz, 1993; Ciani and Maccarelli, 1997), the potential positive role they play in the organoleptic characteristics of wine has been emphasized in numerous studies

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(Fleet, 2003). Metabolic interactions between non-*Saccharomyces* and *S. cerevisiae* wine yeasts during fermentation could positively or negatively interfere with the growth and fermentation behaviour of yeast species, particularly *S. cerevisiae*. It has also been noted that the presence/absence of *S. cerevisiae* differs according to each plant and grape cluster (Pretorius et al., 1999). For this reason it is not always possible to obtain the same product from spontaneous wine fermentation. This problem is being solved at present by the use of commercial strains in the fermentation process, to the detriment of the wine's autochthonous character. On the other hand, the widespread use of LSA has led to a leveling of the aromatic characteristics of wines produced with different cultivars. Today is increasing the demand for selected native yeasts starter that could enhance the sensory characteristics peculiar of the wine. The purpose of this work was the identification of cultures of native yeast to be used in starter mix for use on a large scale

2. Materials and Methods

2.1 Chemicals

The culture media for the isolation, the reactivation and the growth of yeast strains were purchased from Oxoid (Hampshire, UK). Chemical reagents and solvents are all of analytical grade and were from Sigma- Aldrich (St.Louis, MO, USA). The SPME fibers PDMS- 100µm (polydimethylsiloxane), was from Supelco (Bellefonte, PA, USA).

2.2 Sampling

In order to evaluate the change in total polyphenols and antioxidant activity, the Coda di Volpe grapes were harvested in two different stages of maturation, in the last week of September and in the second week of October respectively. Figure 1 reported the experimental protocol diagram.

2.3 Analysis of total polyphenols

Total polyphenol content was determined using the Folin-Ciocalteu reagent (Waterman and Mole,1994) following a micro scale as described by Arnous et al. (2002). The absorbance at 750 nm was recorded, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as standard and expressed as mg gallic acid equivalents/L (GAME).

2.4 HPLC analysis of phenolic compounds

Phenolic compounds analysis was performed by HPLC/UV with a C18 column Hypersil (ThermoElectron Corporation, Bellefonte, PA, North America). The injected sample volume was 20 µL. Peak identifications were confirmed from retention times and direct comparison to pure standards. The solvent flow rate was 0.9 mL/min and the mobile phase was a four-step linear solvent gradient system(0–5 min, 10 % B; 5-40 min, 45 % B; 40–45 min, 100 % B; 45-50 min, 100 % B; 50-55 min, 10 % B)using 2 % acetic acid in water as solvent A and 0.5 % acetic acid in 50 % acetonitrile as solvent B. UV detection was carried out at 280 nm (Katalinić et al., 2010).

2.5 Antioxidant Activity of grape skin extracts

Antioxidant properties of grape skin extracts were determined as free radical-scavenging ability (DPPH method), reducing power (FRAP method), Fe2+-chelating ability of plant extracts, and ability to prevent oxidation of linoleic acid (Katalinić et al., 2010).

2.6 Isolation of yeasts

To isolate the different yeast populations, serial dilutions of Coda di Volpe grape from sealed bag were prepared. 1 mL of must was used to prepare Ringer solution serial dilutions and appropriate dilutions were plated on two different solid agar media: WL-nutrient and Lysine agar. On WL medium, yeast species have been distinguished by different colony morphologies and colours. The isolation of non-*Saccharomyces* species has been carried out on Lysine medium, that does not allow the growth *Saccharomyces spp (*Cavazza et al 1992). After 4 days of incubation at 25 °C, the colonies were enumerated to determine the order of population. Five colonies were isolated randomly from the plates and stored at 4°C for physiological and technological characterization.

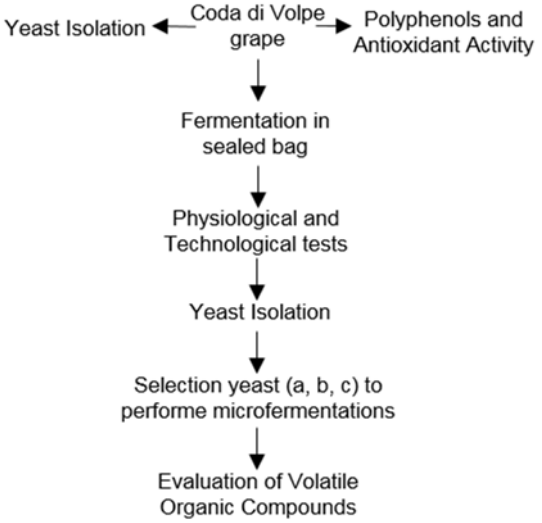

Figure 1: experimental protocol diagram

The culture yeasts were identified according to their morphological aspect (elliptical and lemon shaped) and biochemical test API 20C Aux (bioMerieux, France).

2.7 Determination of the yeast fermentative power

The must (100mL) after pasteurization (100 °C for 30 min) was inoculated with a 1 % (v/v) microbial biomass suspension (24h growth). The must fermentation tests were carried out in shake-flasks incubated at 28 °C. The flasks weight decrease was measured every day, until a constant weight has been reached and maintained for three days (Zambonelli, 1998).

2.8 Yeasts selection for the resistance to sulphur dioxide

The tests were carried out on Coda di Volpe must which was pasteurized and inoculated with a 1 % microbial biomass suspension (24h growth). Potassium metabisulfite (MBK) was used as the source of sulphur dioxide and the yeasts resistance at concentrations of 100 and 250 mg/L was checked. The tests were performed at 28 °C for 7 days (Costanti et al., 1998).

2.9 Inoculated fermentations in laboratory-scale

Three yeasts (YOCMCVWL4, YTECMCVWL4, YTECCVWL7), selected on the base of technological traits were inoculated in must in single and different combination. Inoculated fermentation assay was performed in 1L Erlenmeyer flasks filled with 500 mL of Coda di Volpe grape must with 10% MBK. Grape must characteristics were: 21,5 °Brix, pH 3,2. Each strain was inoculated in grape must at a concentration of 10^6 cells/mL. The fermentation was performed at 20°C and the fermentative course was monitored by measuring weight loss, determined by carbon dioxide evolution during the process. At the end of the process, the wine samples, immediately, were analyzed by SPME-GC/MS to evaluate the volatile compounds.

2.10 Analysis of volatile compounds by HS-SPME-GC/MS

The SPME fiber (PDMS-100µm, polydimethylsiloxane) was conditioned according to the manufacturer's recommendations prior to its first use (Pawliszyn 2009). To a 20 mL Headspace vial was added 5 mL of wine samples, together to 3g of NaCl and octan-3-ol, in hydro-alcoholic solution (1/1, v/v) at 100 µg/L, as Internal Standard. The solution was homogenized with a vortex shaker and then loaded onto a Gerstel autosampling device. The program consisted of swirling the vial at 250 rpm for 5 min at 40 °C, then inserting the fiber into the headspace for 30 min at 40 °C as the solution was swirled again, then transferring the fiber to the injector for desorption at 240 °C for 30 min.

2.11 GasCromatography-MassSpectrometry

Gas chromatography analysis were carried out using a 7890 Agilent GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer equipped with a Gerstel MPS2 autosampler. The capillary column employed was a HP-Innowax (Agilent technologies) (30 m x 0,25 mm id. 0,50 µm film thickness) and the carrier gas was Helium. Splitless injections were used. The initial oven temperature was set to 40 °C for 1 min.
The temperature was increased in four steps: 40–60 °C at 2 °C/min; 60–150 °C at 3 °C/min, 150–200 °C at 10 °C/min and 200-240 °C at 25 °C/min; the final temperature was maintened for 7 min. The injector, the quadrupole, the source and the transfer line temperature were maintained at 240 °C, 150 °C, 230 °C and 200 °C, respectively. Electron ionization mass spectra in full-scan mode were recorded at 70eV electron energy in the range 40–300 amu. Peaks were identified using both the NIST 98 and Wiley libraries. Quantification was performed by using the relative concentration in µg/L of the Internal Standard, calculated as the ratio between each compound area and the internal standard area. The samples were analyzed in triplicate and blank runs were made by using an empty vial every two analysis (Sorrentino et al., 2013).

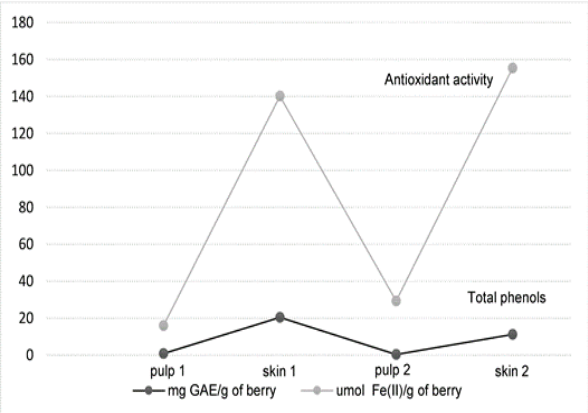

Figure 2: total phenols and antioxidant activity relative to skin and pulp grape berry in two different time sampling

3. Results and Discussion

3.1 Total polyphenols and antioxidant activity

The data reported in Fig.2 show the content of total polyphenols and antioxidant activity in Coda di Volpe berry grapes. They show that the amounts was highest in the skin respect to pulp berry. These results were confirmed in all samples collected to the different times (last

week of September and the second week of October). In addition, experimental data have shown that polyphenols were higher in the skin of the grape berry harvested in the first sampling while the antioxidant activity was higher in the berry in second. The HPLC analysis on grape skin (data not shown), confirmed the presence of catechins and epicatechins peaks in both sampling, this data was in according to literature (Katalinić et al., 2010).

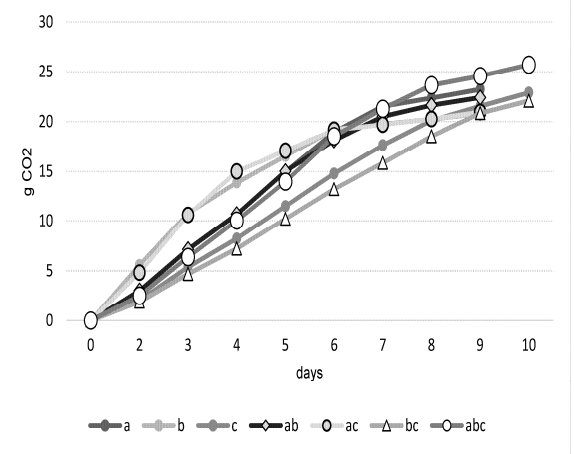

Figure 3: fermentative power of single and mix yeast selected, evaluated by loss in CO_2

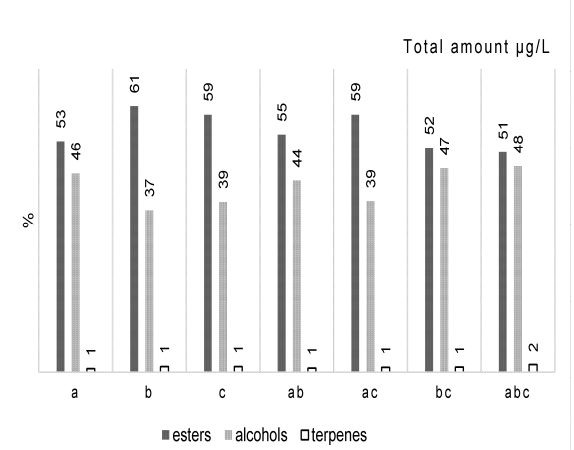

Figure 4: principal VOC classes on Coda di Volpe wine, determined by SPME-GC/MS, reported in percentage.

a: YTECCVWL7; **b**: YTECMCVWL4; **c**: YOCMCVWL4; **ab**: YTECCVWL7+ YTECMCVWL4; **ac:** YTECCVWL7+ YOCMCVWL4; **bc**: YTECMCVWL4+ YOCMCVWL4; **abc**: YTECCVWL7+ YTECMCVWL4+ YOCMCVWL4

3.2 Monitoring of yeast populations on Coda di Volpe grapes

The results of microbiological analyses on the grapes showed that in the first sampling the epiphytic microflora was around $3*10^3$ cfu/g on WL agar and $2*10^4$ cfu/g on Lysine medium, while on the second sampling the load of yeast population was respectively $5*10^5$ cfu/g and $6*10^5$ cfu/g. After enumeration, from the plates contained from 15 to 300 colonies were isolated randomly five colonies. These were stored at 4°C for identification and technological characterization. After physiological screening, were selected yeasts with elliptical and lemon shaped (Martini et al., 1996). The yeasts selected were *S.cerevisae* YTECCVWL7 (a) and YOCMCVWL4 (c), non-*Saccharomyces* YTECMCVWL4 (b), they were used in technological tests and to performe microfermentations.

3.3 Technological characterization

On yeast selected, was carried out the technologic test in order to ascertain resistance of the indigenous yeasts to sulphur dioxide. Sulphur dioxide is the most widely antiseptic agent used in wine cellars to control the growth of spontaneous microflora during fermentation. For our selection, we checked the resistance at two different concentrations (100 mg/L and 250 mg/L). The results demonstrated that most of the yeasts were resistant to SO_2 concentration 100 mg/L. Our data, revealed that many indigenous yeast belonged to non-*Saccharomyces* genera showed resistance to SO_2, to despite the literature data (Romano and Suzzi, 1993; Henick-Kling et al., 1998) that suggest the lack of such resistance in many yeast non-*Saccharomyces* genera. The resistance to sulphur dioxide could be caused by a “selective pressure” of sulphur levels used on grapevines throughout the years. Moreover, a significant number of indigenous yeasts non-*Saccharomyces* can grow after the addition of the usual dose of SO_2 until they die of ethanol intoxication after three or four days from the beginning of fermentation (Fleet, 2003). Based on the results, we chosen three yeasts that showed best performance. These yeasts were used to perform fermentative power test along and in mix in Coda di Volpe must. The results (Fig.3) showed that all yeast as along as mix, were able to start and complete the wine fermentation. Moreover, the **abc** mix showed the best fermentative power as demonstrated by higher CO_2 production.

3.4 Volatile compounds

The results obtained from SPME-GC/MS analysis on the product of microvinification in batch, were reported in Table 1. The Esters are considered important to the sensory properties of wines, contributing with positive aroma, essentially with fruity notes (Pereira et al., 2014). The results (Fig.4) showed that esters were one of the most abundant groups (about 55%) as they are secondary aromas. While, the higher alcohols may be present

in healthy grapes, but seldom occur in significant amount, they are essentially formed either from sugar catabolism or from amino acid decarboxylation and deamination and they have high perception threshold. They commonly account for about 50% of the aromatic constituents of wine, excluding ethanol. Moreover, the terpenes are varietal compounds present in grapes, especially in skins, o arise from glycosil precursors and they have low perception threshold.

Table 1: Volatile compounds identified in microvinification with single and different combination of selected yeasts. Each value is expressed in µg/L and is the mean of three replicates ± sd (standard deviation)

	a	b	c	ab	ac	bc	abc	odor [a]
	µg/L sd	µg/L sd	µg/L sd	µg/L sd	µg/L sd	µg/L sd	µg/L sd	
Esters (14)								
ethyl acetate	3166,6 ± 241,	1134,6 ± 118,2	871,4 ± 20,3	3110,3 ± 44,9	1840, ± 4,1	2066,1 ± 153,	2704,3 ± 54,4	fruity
isobutyl acetate	7,7 ± 0,6	19,8 ± 2,1	nd	19,4 ± 0,2	9,6 ± 0,02	nd	8,90 ± 0,2	fruity
Ethyl butanoate	121,1 ± 9,2	90,4 ± 9,4	92,2 ± 2,1	178,0 ± 2,5	117,2 ± 0,2	126,5 ± 9,4	123,3 ± 2,4	floral,
ethyl	9,27 ± 0,7	10,54 ± 1,1	8,26 ± 0,1	16,50 ± 0,2	11,75 ± 0,03	15,76 ± 1,1	9,34 ± 0,1	sweet fruit
Ethyl	1153,3 ± 87,9	1284,8 ± 133,9	760,1 ± 17,7	1693,1 ± 24,4	1112, ± 2,5	1100,3 ± 81,9	1034,6 ± 20,8	fruity
Hexyl acetate	8,5 ± 0,6	63,6 ± 6,6	6,7 ± 0,1	26,1 ± 0,3	40,2 ± 0,01	7,1 ± 0,5	9,2 ± 0,1	herbaceou
ethyl lactate	nd	nd	7,8 ± 0,2	nd	112,0 ± 0,3	54,2 ± 4,1	nd	Milk,butter
Ethyl octanoate	6493,1 ± 495,	9094,5 ± 947,9	6364, ± 148,	8058,5 ± 116,	7898, ± 17,8	6422,6 ± 478,	5638,2 ± 113,	sweet fruit
isoamyl acetate	783,2 ± 59,7	1332,8 ± 138,3	539,5 ± 12,6	1322,2 ± 19,1	1013, ± 2,3	940,6 ± 70,0	803,8 ± 16,1	banana
Ethyl	3612,17 ± 275,	4112,4 ± 301,3	3172, ± 74,1	4270,2 ± 61,7	2767, ± 100,	3400,6 ± 250,	2560,7 ± 51,5	oily, fruity
diethyl	13,7 ± 1,1	16,3 ± 1,7	18,6 ± 0,4	17,6 ± 0,2	38,1 ± 0,1	45,3 ± 3,3	29,8 ± 0,6	fruity
ethyl 9-	848,8 ± 64,7	2496,1 ± 260,1	2795, ± 65,3	1720,8 ± 24,7	3298, ± 70,4	1267,5 ± 94,7	1075,9 ± 21,5	-
Phenethylaceta	351,4 ± 26,8	306,4 ± 10,1	328,8 ± 7,6	443,5 ± 9,1	491,5 ± 20,3	349,7 ± 25,5	403,5 ± 8,2	flowery
ethyl laurate	345,5 ± 26,3	410,6 ± 42,8	269,2 ± 6,2	482,2 ± 6,9	188,4 ± 10,2	337,8 ± 30,3	271,5 ± 5,7	sweet
Alcohols (8)								
Isobutanol	733,7 ± 55,9	962,7 ± 100,3	303,3 ± 7,1	1111,5 ± 16,1	790,3 ± 1,7	736,4 ± 54,8	857,5 ± 17,6	fusel
isoamyl alcohol	10629,7 ± 810,	10340, ± 1077,	8088, ± 188,	13202, ± 190,	9897, ± 22,4	11728, ± 873,	10553, ± 212,	fusel
4-methyl-1-	nd	10,5 ± 1,1	5,5 ± 0,1	nd	nd	6,4 ± 0,4	3,9 ± 0,1	almond
3-methyl 1-	nd	15,3 ± 1,6	17,6 ± 0,4	13,5 ± 0,2	11,9 ± 0,03	16,9 ± 1,6	10,7 ± 0,2	vinous
1-hexanol	53,1 ± 4,05	6,8 ± 0,7	38,7 ± 0,9	52,5 ± 0,7	9,1 ± 0,02	nd	45,8 ± 0,2	herbaceou
2-ethyl hexanol	nd	nd	4,3 ± 0,1	nd	nd	nd	8,7 ± 0,2	mushroom
2,3-butandiol	33,6 ± 2,5	nd	nd	18,3 ± 0,2	nd	17,6 ± 1,3	9,4 ± 0,2	butter
phenethyl	3122,59 ± 238,	1084,6 ± 113,1	1621, ± 37,8	2838,3 ± 41,1	1878, ± 4,2	2147,2 ± 159,	2256,5 ± 45,1	rose
Acids (3)								
acetic acid	232,9 ± 17,7	31,0 ± 3,2	24,8 ± 0,6	255,4 ± 3,6	69,1 ± 0,1	62,9 ± 4,6	237,9 ± 4,9	vinegar
octanoic acid	391,6 ± 29,8	65,3 ± 6,8	386,4 ± 9,1	12,2 ± 0,1	869,5 ± 1,9	475,1 ± 35,3	512,6 ± 10, 2	fatty
decanoic acid	352,1 ± 26,8	69,3 ± 7,2	486,6 ± 11,4	128,2 ± 1,8	894,6 ± 2,1	533,3 ± 39,7	504,4 ± 10,1	fatty
carbonyl comp								
acetoino	29,5 ± 2,2	38,9 ± 4,1	nd	61,0 ± 0,8	42,5 ± 0,1	nd	nd	butter
acetaldehyde	76,3 ± 5,8	14,0 ± 1,4	30,4 ± 0,7	61,3 ± 0,8	47,2 ± 0,1	25,6 ± 1,9	93,9 ± 1,8	ripe apple
Terpenes (6)								
beta farnesene	11,1 ± 0,8	16,3 ± 1,7	17,6 ± 0,4	16,6 ± 0,2	27,5 ± 0,1	20,8 ± 1,5	22,4 ± 0,4	citrus
alfa-farnesene	5,2 ± 0,4	12,9 ± 1,3	11,4 ± 0,2	11,9 ± 0,1	17,6 ± 0,11	19,7 ± 1,4	14,6 ± 0,3	wood
nerolidol	93,7 ± 7,1	186,6 ± 19,3	175,2 ± 5,1	180,1 ± 2,1	129,6 ± 0,3	151,3 ± 11,2	173,6 ± 3,4	wood
2,3-	60,7 ± 4,6	97,7 ± 5,6	92,5 ± 3,7	80,0 ± 0,4	96,9 ± 0,3	75,3 ± 6,5	143,5 ± 4,1	swee
farnesol	85,3 ± 9,3	98,2 ± 4,6	94,1 ± 3,8	80,2 ± 0,6	78,1 ± 0,3	80,3 ± 9,7	112,7 ± 3,2	flower
beta citronellol	nd	nd	14,5 ± 0,3	nd	9,3 ± 0,02	10,6 ± 0,7	39,2 ± 0,7	citrus

[a] Based on flavornet (www.flavornet.org) and pherobase (www.pherobase.com) online databases
nd: not detected

In conclusion, we selected the mix **abc**, to apply in future winemaking process on large scale, because it showed the best fermentative power and a balance in the amount of esters and alcohols products, and a higher content of terpenes (2%), being these volatile compounds classes responsible to modulation of wine aroma and flavor.

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