Definitive Evidence for the Actual Contribution of Yeast in the Transformation of Neutral Precursors of Grape Aromas

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Experiments were designed to demonstrate the actual contribution of yeast in the formation of the primary aroma during the vinification of neutral grapes. Ruché was chosen as the model wine to study because of its unique fragrance. A yeast strain specific for Ruché was selected using a new and rapid isolation method for red wines. The results of this study can be summarized as follows: Skins from nonaromatic white or red grapes apparently contain most of the primary aroma compounds that are revealed in the must only after contact with yeast cells under defined conditions. Similar results were obtained with the pulp and seeds fractions; however, the olfactory notes, although well characterized, differed from those obtained with skins alone. Clarification, filtration, and centrifugation of the pulp and seed fractions or sonification of the skins produce different and well-characterized olfaction notes during the contact with yeast. The primary aroma of nonaromatic white and red grapes contained in the skins can be revealed within 24−48 h of yeast contact in a synthetic nutrient medium (SNM). The primary aroma precursors extracted from the skins with methanol, water-saturated butanol, or aqueous buffer at pH 3.2, concentrated and eluted from a C18 resin column, can be transformed to the free form wine aroma markers within 6 h of contact with yeast cells in SNM. By contrast, prolonged maceration of the skins in aqueous alcoholic buffer at pH 3.2 or 1.1, at 50 or 70 °C did not release primary odors typical of wine. The individual primary aroma compounds, identified by GC-MS analysis in Ruché wine samples or in Ruché skin-yeast-SNM samples, could not explain the complexity of the typical Ruché wine odor. Only odors common to many wine varieties were identified by GC−olfactometry analysis.

Keywords: *Selected yeast; primary aroma; wine variety*

INTRODUCTION

The identity of many aromatic markers produced by some grape vines (primary aroma or fragrances) as the free form and as precursors are already known. For example, the berries of Moscato and Malvasia vines are known to be particularly rich in terpenes (linalool, α-terpineol, nerol, geraniol, hotrienol, pyran, and furan forms of the linalool oxides) (*1*−*4*). Furthermore, some terpenes, as nonvolatile nonfragrant glycosidic derivatives (aromatic precursors), have been encountered frequently and in variable concentrations in all varieties of *Vitis vinifera* examined. In the case of the aromatic varieties, the Traminers were found to have additional precursor markers in the form of volatile esters of the dominant terpenes, geraniol, nerol, and diols (i.e., as the methyl esters of *trans*-geranic and farnesoic acids, farnesol, and two α-farnesene isomers) (*5*), the Muller-Thurgau were found to have free terpenes (ho-diendiol I, linalool), and bound terpenes (α-terpineol, linalool oxides, 8-OH-linalool, *p*-menth-1-ene-7,8-diol) (*6*), while Riesling was found to have the 2-ethyl-3-methylmaleimide derivative (*7*).

The fragrant free form of the terpenes (linalool, α-terpineol, nerol, geraniol) can be released enzymatically from grape mono- and diglucosides by the simultaneous action of plant *β*-glucopyranosidase and yeast periplasmic *β*-D-apiofuranosidasis, L-arabinofuranosidasis, and α-L-rhamnopyranosidasis. The yeast enzymes apparently act first to release the terminal sugar from the disaccharides (*1*−*4*, *8*−*19*) followed by the action of the plant glycosidases. How these enzymes fit into the picture is a paradox considering the fact that plant *β*-glucosidase activity is strongly inhibited by the concentration of glucose found in the must of Moscato grapes, yet the must is found to be replete with free terpenes immediately after grape crushing. By contrast, according to Darriet et al. (*9*), Cordonnier et al. (*16*), and Delcroix et al. (*20*), the yeast *β*-glucosidases are only 20% inhibited in the presence of 100 g/L of glucose and 10% inhibited in the presence of 15% alcohol, while 50 mg/L of SO_2 does not have any effect. Furthermore, at the pH of must (2.8−3.8), the stability of plant or fungal *β*-glucosidase is very low (5% at pH 2.8) contrary to what appears to be the case for yeast periplasmic *β*-glucosidase. Analogous to the terpene glycosides of Moscato, the existence of glycosides of various other aromatic markers have also been identified (*21*).

Hock et al. (*22*), confirming the investigations of Drawert et al. (*23*) and Fagan et al. (*24*), found that in a synthetic medium enriched with amino acids, some strains of *Saccharomyces rosei* (*Torulaspora del-*

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brueckii), Kloeckera apiculata, *Metschnikowia pulkerrima*, and *Torulopsis stellata (Candida stellata)* were able to produce up to 1572 μg/L of linalool, up to 1176 μg/L of β-myrcene, 1230 μg/L of limonene, and 1745 μg/L of α-terpineol, while other strains of *S. cerevisiae, S. bayanus and K. apiculata* produced up to 2350 μg/L of farnesol. Drawert et al. (*23*) pointed out that monoterpene biosynthesis does not necessarily depend on special precursors and found that culturing a strain of *Kluyveromyces lactis* at higher temperatures (up to 27 °C) and higher asparagine concentrations increased the production of citronellol. In addition, it has been demonstrated that yeast can contribute to the transformation of geraniol to citronellol (*23*) or to geranyl acetate and the conversion of citronellyl acetate and nerol to neryl acetate (*25*).

It has also been demonstrated that other wine aroma compounds can be released by the activity of wine yeast lyase (*26*, *27*). This class of aroma compounds includes cysteine conjugates, which upon hydrolysis of the thiol ester releases an odor characteristic of box wood or black currants, but with a very low olfactory threshold.

Allen et al. (*28*) found that the odors of grass and capsicum (green bell peppers) was due to methoxypyrazines, while Darriet et al. (*26*), Dubourdieu (*29*), Dubourdieu et al. (*30*, *31*), Tominaga et al. (*27*, *32*, *33*), and Peyrot des Gaschons et al. (*34*) showed that the fragrance of box wood, black currants, tomatoes, and passion fruit leaves was due to a group of thiol compounds that included 4-methyl-4-mercaptopentan-2-one and the 3-mercaptohexyl acetate. They found that the release of these thiol compounds from a precursor present in the must was a function of fermentation conditions and the strain of yeast used.

The origins of the aromas described should not be considered as isolated cases restricted to the varietals cited, but it can be reasonably assumed that an analogous situation can occur in other grape varieties whether or not they produce primary aromas.

Since that concept has already been submitted for critical review (*35*−*37*), the question that springs to mind is the following: are the glycosidic or nonglycosidic precursors in a particular grape (the substrate for aroma transformed by yeast) unique to that vine, or are they common constituents among genetically related grape vines? For example, it is known that the methoxypyrazines are common to both Cabernet Sauvignon (*38*) and Sauvignon Blanc (*28*), two genetically related varietals. In addition to the known terpenes, compounds such as 1,1,6-trimethyldihydronaphthalene have been identified in Chardonnay (*39*), monoterpenes and norisoprenoids in Silvaner, Weissburgunder, and Rulander (*40*), and vitispirane in Riesling (*41*). What is still unresolved is the molecular structure of their respective precursors and the biological source or the biochemical mechanisms involved in their transformation. It appears reasonable to ask specifically: does yeast play an active role during alcoholic fermentation in the transformation of potentially aromatic compounds? In addition, can this role be identified with the chemical or enzymatic methods proposed by some investigators (*19*, *42*)?

We cannot casually dismiss the fact that a must prepared from a nonaromatic grape lacks an obvious smell. But analysis of this type of must by gas chromatography reveals classes of compounds some of which are common to musts of all grape varieties. In addition, after the yeast fermentation is completed, these putative nonaromatic precursor compounds are transformed to a pool of fragrant substances with aromas characteristic of the varieties in question. Therefore, despite a paucity of knowledge concerning the biochemical mechanisms utilized by *S. cerevisiae* in the formation of a characteristic wine aroma profile, it cannot be denied that yeast is the biological driving force behind the transformation of aromatic precursors during the alcoholic fermentation.

The research presented in this paper provides evidence that, for the first time, unequivocally demonstrates the active role wine yeast plays in the formation of typical wine aromas derived from compounds found in white and red variety classified as neutral. Pinot Noir and Ruché grapes were chosen for this study. Ruché represents a particularly useful model for study because its characteristic odor is unlike any other aromatic descriptors currently used in enology.

MATERIALS AND METHODS

Grapes. Sound ripe Pinot Noir grapes (from the Aosta Valley) and Ruché grapes (from Castagnole Monferrato) were selected and harvested by hand with utmost care under stringent sanitary conditions. Harvested grapes were stored frozen at −20 °C, and when needed, thawed slowly overnight at 4 °C. Frequently, freshly picked grapes were used directly in this study.

Media. Synthetic nutrient medium/complete (SNM/c), prepared as described by Delfini et al. (*43*), was supplemented with glucose (100 g/L), ammonium sulfate (100 mg/L), ammonium phosphate (100 mg/L), tartaric acid (3 g/L), malic acid (3 g/L), pH 3.20. Synthetic nutrient medium/minimal (SNM/m) is essentially the same as SNM/c without glucose or with only 5 g/L glucose (designated as SNM/m5g).

Yeasts. *S. cerevisiae*, strain AM99, was specifically selected for Pinot Noir as described by Delfini et al. (*35*), *S. cerevisiae*, strain BR94, was specifically selected for Ruché, and *S. cerevisiae*, strain S46c, was selected as a generic strain for red wines. The yeast cultures were grown on YM-agar (*44*) or in SNM/c from cultures stored at +4 °C on YM-agar. The respiratory deficient mutant (HRO^-) of *S. cerevisiae*, derived from strain AM99, was provided by Frontali et al. from the Institute of Genetic, University "La Sapienza", Rome.

Preparation of Grape Skins. Sound grapes were squeezed gently by hand to expel the pulp and seeds. The skins were either used whole, split in two, minced into small fragments, homogenized in a Waring blender, or sonicated. The skin preparations were washed by centrifugation and used as described in the text.

Isolation of Volatile Compounds. In all experiments involving yeast incubations, the culture supernatants were harvested and subjected to three consecutive liquid−liquid extractions by sonication with methylene chloride according to the procedure of Cocito et al. (*45*). The organic phases were collected and concentrated 1000-fold by evaporation in a rotary evaporator at 30 °C. Whatever residual solvent was present in the spent aqueous fraction was allowed to evaporate spontaneously at room temperature. The aqueous fraction was also subjected to olfaction analysis. Volatile compounds were also collected by solid-phase microextraction (SPME) methods. This method was used to identify volatiles in the headspace (HS) of the incubation flask. This procedure was carried out by suspending a 65 mm poly(dimethylsiloxane)/Divinylbenzene polymer for polar volatiles (SPME Fiber Assembly, SUPELCO) into the HS of the flask being sampled, for 30 min at 25 °C.

Identification of Volatile Compounds. The volatile compounds isolated from the samples by liquid−liquid extraction with methylene chloride or by SPME adsorption were identified by mass spectrometry coupled to a gas chromatograph (GC Varian Star 3400CX joined 2000 MS/MS to Varian Saturn) using a column Supelcowax 10 TM, 30 m × 0.25 mm, 0.25 μm film as described previously (*45*).

GC−Olfactometry Analysis. The aromatic markers were analyzed by a GC (Varian 3600) as described above but equipped with an FID and an olfactory detector outlet SGE. Both types of samples (liquid−liquid and SPME) were subjected to olfaction analysis.

Olfaction Analysis. The identification of the odors produced in the Pinot Noir and Ruché experimental samples was performed by five expert tasters experienced with Pinot Noir or Ruché wines. The olfaction analysis was done directly in the tasting glass in which the plant material interacted with the yeast. In some experiments, a direct comparison was made with the corresponding wines. The preference test was performed with a nonparametric card by 14 experienced expert wine tasters. The results were statistically analyzed using the Friedman Test.

Isolation of Yeast Strains Specific for Red Wine (Experiment I). The initial selection involved yeast colonies isolated from Ruché grapes, musts, and wines according to procedures developed by Delfini (*43*). Selection involved using synthetic nutrient media, such as YM-agar media (*46*), to verify the taxonomic characteristics of the isolates. Subsequently, growth in SNM/c (*43*) was used to determine the more important enological characteristics of the isolates, such as: alcohologenic capability, rate of sugar consumption, sugar/alcohol conversion coefficient, rate of alcohol production and growth curves, ability to produce acetic acid, lactic acid, pyruvic acid, glycerol, acetaldehyde, sulfur dioxide, hydrogen sulfide, ability to degrade malic acid, ability to grow at lower temperatures, level of resistance to sulfur dioxide, and ability to produce pleasant or unpleasant odors. Strains selected from the initial screening showing promising characteristics were used to inoculate Ruché grape must prepared for red wine production (in the presence of skins). To emulate the actual technological conditions used for red wine fermentation, the following protocol was used.

A sufficient amount of intact fresh berries (or berries stored frozen at −20 °C and slowly thawed overnight at 4 °C) were detached from their stems with scissors. The berries were batch-washed under running tap water to remove any adhering extraneous matter (soil, grape juice, sugars, etc.) and to rinse off contaminating microorganisms. The washed berries were allowed to drain on sheets of paper toweling and then distributed equally by weight, 100 g per 200 mL fermentation flask. The grapes were gently crushed by hand or with a pestle directly inside the fermentation flasks. Each fermentation flask was inoculated with 5×10^6 cells/mL from a 3-day-old culture of initial isolates that had the requisite characteristics. Daily measurements were made for the determination of growth and alcohol production. This was done by weighing the flasks according to the method of Delfini et al. (*37*). Individual flasks were fitted with a silicon stopper into which was inserted a Pasteur pipet bent at a 170−180° angle. This device allowed gas to escape while preventing the entry of air-borne contamination (*37*). The open capillary end of the Pasteur pipet established a semianaerobic environment, essentially very similar to what actually occurs in a fermentation tank. The wines derived from this procedure, referred to as microvinification samples, were first analyzed by olfaction alone, to identify the best 10−15 strains. Subsequently, a complete organoleptic analysis narrowed the choices to the best 5−6 strains. Two replicates per yeast clone were prepared for this evaluation. The olfaction analysis of the microvinification samples was performed by five expert tasters experienced with Ruché wine. After the assessments were made by each individual taster, the results were discussed and a group consensus was reached as to which were the preferred strains.

When larger volumes of wine were required for a more complete chemical and organoleptic analysis, the following procedure was followed: the strains, which were deemed preferable by the tasters, were used to inoculate several batches of approximately 5 L of Ruché grape-must prepared from 8 kg of berries. The berries were prepared as described above for the microvinification samples. This yeast selection procedure can be called macrovinification and can be used with berries from different vintages to test the performance of the selected strains as a function of grape quality and climate variability.

Identifying the Source of Varietal Odors (Skin, Pulp, Seeds) Produced during Yeast Fermentation (Experiment II). Grape skins, pulp, and seeds of Pinot Noir and Ruché were separated as described. The skins were left whole or fragmented or homogenized and centrifuged or sonicated and centrifuged. The pellets and respective supernatants were assessed separately. Control samples of crushed whole berries, whole skins, fragmented skins with or without yeast or potassium sorbate were also prepared (see the fraction description in Table 3). The pelleted material was suspended in sufficient SMN/m5g to equal the volume of the supernatant samples, placed in standard wine glasses, and capped with aluminum foil. The appropriate samples were inoculated with $40-50 \times 10^6$ cells/mL of either strain AM 99 (Pinot Noir specific) or strain BR 94 (Ruché specific). Olfaction analysis was performed daily for 10 days. The possibility of contamination in the samples was monitored daily by microscope examination.

Incubation Conditions for the Optimal Expression of Primary Aromatic Markers from Skins (Experiment III). For the current study, the following protocol was followed: 15 g of Pinot Noir or Ruché skins were minced and suspended in 30 mL of SMN/m in a standard size (225 mL) wine tasting glass. Five glasses were prepared with Pinot Noir skins; another five received Ruché skins. The wine glasses containing the Pinot Noir skins were inoculated with 50×10^6 cells/mL of strain AM99, the Pinot Noir specific strain (sample 3); similarly, wine glasses with Ruché skins were inoculated with 50×10^6 cells/mL of strain BR94, the Ruché wine specific strain (sample 4). Both yeast cultures were in the exponential phase of growth when added. Each wine glass was covered with aluminum foil and incubated. The controls, with either Pinot Noir (sample 1) or Ruché (sample 2) skins, remained uninoculated and were incubated for 48 h at 25 °C. Some of the wine glasses were incubated for 48 h at 25 °C (samples 3 and 4) or at 50 °C for 7 days (samples 5 and 6) or at 70 °C for 7 days (samples 7 and 8). In addition to investigating the effect of temperature on aroma production, the presence of ethanol on aroma production was also investigated. Some of the inoculated samples contained 10% ethanol and incubated at 25 °C for 7 days (samples 9 and 10).

Acid Hydrolysis (Experiment IV). A total of 15 g of Ruché grape skins were fragmented, rinsed in distilled water to remove residual must juice, suspended in 30 mL of citrate buffer at pH 1.1 (sample 1) and 3.2 (sample 2), and incubated at 25 °C for 50 days. Duplicate samples were prepared for each pH treatment. One of the pair was incubated aerobically (a) in flasks closed with a bent Pasteur pipet, and the mate was incubated anaerobically (b). Anaerobic conditions were established by bubbling N_2 through the flask for 15 min, and then the flask was closed off with the attached Muller valve. Anaerobic conditions are inherently inhibitory to the growth of mould contaminants. To determine the effect of growth of yeast contaminants, samples were prepared with and without the addition of 400 mg/L potassium sorbate plus 100 mg/L SO_2 and monitored repeatedly by microscopic examination. The olfaction analysis was performed daily during the first week and then weekly until the experiment was concluded (50 days).

Aroma Profile of Ruché Wine in Comparison with Other Red Wines (Experiment V). In the preparation of microvinifications for this study, 200 g of berries were removed from the stalks and crushed by hand. The whole must, complete with skins, was placed in a 500-mL sterile container, inoculated with 3×10^6 yeast cells/mL (strain BR94), and covered with aluminum foil. At the end of the fermentation (determined by weighting), the wine was decanted from the pomace, centrifuged, and stored at −20 °C until assayed.

Wine Odor Released by Skin−Yeast Contact (Experiment VI). Fragmented grape skins were prepared from different aromatic and nonaromatic grape varieties: (I) white grapes: Arneis, Cortese, Erbaluce, Favorita and Moscato; (II) red grapes: Barbera, Cabernet Sauvignon, Croatina, Dolcetto,

Nebbiolo, Pinot Gris, Brachetto, Merlot and Ruché. The grape skins were prepared as described. Each wine glass was inoculated with 50×10^6 cells/mL of the generic strain S46c after the yeast culture was in the logarithmic phase of growth. The wine glasses were covered with aluminum foil and incubated for 48 h. The positive controls were prepared with whole must and the negative controls remained uninoculated. At the end of the incubation period, the experimental samples and the corresponding wines were compared by olfactory analysis.

Isolation and Identification of Wine Odor Compounds Released from Grape Skin Precursors by the Activity of Yeast (Experiment VII). (*1) Preparation of Aroma Precursor Extracts*. In addition to extracting aroma precursors from grape skins, this procedure was designed to remove sugars and other assimilable compounds from the precursor extract as a way of minimizing yeast fermentation activity when exposed to the extract, and to facilitate passage through the C18 column. A total of 125 g of Ruché skins were obtained by crushing the grapes manually and expelling the pulp and seeds as described above. For the isolation of aroma precursors, the harvested skins were split in two parts and soaked in 125 mL of one of the following solvents: (a) water-saturated butanol (a 1:3 mixture of water and butanol was shaken together in a separatory funnel and allowed to separate. The organic fraction was removed and used in the experiment) (b) buffer (Bm): 3 g/L of malic acid in aqueous solution brought to pH 3.2 with 1 N KOH, or (c) methanol. After the sample was stirred for 4 h on a magnetic rotary stirrer, the liquid was decanted and the spent skins were discarded. The volume of the buffer-treated sample (extraction procedure b) was reduced by lyophilization to around 100 mL. Samples extracted with solvents (procedures a and c) were evaporated to dryness in a rotary evaporator under vacuum (refrigerated at −15 °C). The residue was subsequently suspended in approximately 100 mL of Bm.

(2) C18 Resin Treatment. The resuspended samples prepared from solvents a, b, or c described above were first centrifuged at 4000 rpm for 15 min, and the pellets (pt) were washed once by centrifugation with 10 mL of Bm. The pt were stored at −20 °C. Grape skins not soaked ("untreated") in solvents a, b, or c served as controls. The supernatants from the two centrifugations were combined, clarified by filtration through glass wool, and then passed through a C18-(10 g) resin cartridge (Waters, C18 cartridges, Sep-Pak) to eliminate acids, salts, and sugars. The effluents were cycled through a C18-(5 g) cartridge until the effluents ran colorless. The putative precursors were subsequently eluted from the column with methanol, evaporated to dryness in a rotary evaporator, and suspended in 50 mL of SNM/m. This solution was added to the corresponding pt, inoculated with 50×10^6 cells/mL of the BR94 strain of *S. cerevisiae* and incubated for 6 h at 25 °C. Control samples without the addition of yeast cells were similarly prepared. After the samples were incubated, the culture supernatants were supplemented with 2-octanol as the internal standard, extracted with methylene chloride as described, and analyzed by GC-MS chromatography.

RESULTS AND DISCUSSION

Selection of Yeast Strains Specific for Ruché Wine (Experiment I). For the isolation of yeast strains specific for Ruché wine (exp I), 535 isolates were obtained from the grapes and corresponding fermenting musts from three different vineyards and wine producers (symbolized by BR, BORG, and CRI). From the morphological analysis on solid YM-agar media, 365 cultures were selected and used to inoculate SNM/c; 85 isolates produced pleasant aromas in SNM/c and were used subsequently to inoculate Ruché grape-must for the preparation of microvinification samples. From these trials, 20 strains were chosen by olfaction analysis and used to inoculate larger volumes of Ruché grape-must for macrovinification. The wines produced by macrovinification were characterized by the parameters listed in Table 1. The tasting analysis of the macrovinification samples narrowed the preference to the following six strains: BR1, BR14, BR94, BR96, BORG53, CRI29. The tasting analysis was repeated for the preference test. The data listed in Table 2 indicate that five out of 14 tasters gave strain BR 94 a preference score of 6, seven gave it a score of 5, one taster scored it at 4 while another scored it at 3, giving strain BR 94 a preference ranking of 72, followed by strain BR96 at 67 and strain BORG53 at 54 (Table 2). The rankings were verified statistically using the Friedman test at the 5% confidence limits. On the basis of the enological (Table 1) and organoleptic (Table 2) parameters, strain BR94 was chosen as the preferred strain specific for Ruché.

In studying the yeast's contribution to aroma for the improvement of wine quality, it is critical to pay particular attention to the preparation of the scaled down versions of the fermentations (microvinifications). For experimental purposes, whole must preparations are usually carried out using a single batch of crushed grapes which is subsequently distributed evenly among several vessels. The problem lies in the even distribution, i.e., trying to maintain a reproducible skin-to-juice ratio in each replicate fraction. This level of precision is difficult to achieve to be truly representative of what occurs in the winery. However, the new methodology described under exp I ensures a constant ratio between skins and must liquid in all the comparative replicate trials which ensures an equal extraction of aroma compounds from the skins during fermentation. The method also ensures a homogeneous distribution of berries of equivalent qualities among the replicates

Table 1. Enological Parameters from the "Macrovinification Test" Fermented by the Six Strains Listed in Table 2[a]

yeast strains	*R*	alcohol %	total extract g/L	total acidity g/L (as tartaric acid)	volatile acidity g/L	pH
BR1	a	13.40	23.2	6.67	0.48	3.61
	b	13.24	22.4	6.52	0.42	3.60
BR14	a	13.50	22.9	6.52	0.39	3.56
	b	13.24	23.0	6.37	0.42	3.64
BR94	a	13.04	23.6	6.75	0.39	3.60
	b	12.97	23.7	6.75	0.42	3.58
BR96	a	13.50	22.8	6.30	0.36	3.68
	b	13.37	23.9	6.30	0.36	3.60
BORG53	a	13.52	23.7	6.75	0.42	3.60
	b	13.37	23.7	6.75	0.42	3.60
CRI29	a	13.29	22.4	6.45	0.39	3.57
	b	13.15	22.9	6.45	0.39	3.57

[a] *R* = replications.

Table 2. Preference Test Performed by the 14 Tasters (A−P) on the Wines Fermented by the Six Selected Ruché Yeast Strains (Experiment I)[a]

yeast strains	tasters														ranks
	A	B	C	D	E	F	G	H	I	L	M	N	O	P	Σ
(a) BR1	2	2	3	3	6	2	2	4	3	3	2	2	2	5	41[b,c,d]
(b) BR14	1	1	1	6	1	1	1	1	2	2	1	1	1	1	21[a,c,d,e,f]
(c) BR94	5	5	5	5	4	3	5	5	5	6	6	6	6	6	72[a,b,e,f]
(d) BR96	6	6	6	4	5	5	4	6	4	4	5	4	4	4	67[a,b,e,f]
(e) BORG53	4	4	4	2	3	6	6	2	6	1	4	5	5	2	54[b,c,d]
(f) CRI29	3	3	2	1	2	4	3	3	1	5	3	3	3	3	39[b,c,d]

[a] The statistical analysis was performed using the Friedman Test at the 5% significance level. Significant difference versus the corresponding yeast strain/s (a through f).

Table 3. Olfaction Analysis of Experiment II Samples Performed after 3 Days of Fermentation in SNM/m5g[a]

samples with added must components	Pinot Noir	Ruché
Skins after Homogenization and Centrifugation:		
(Bfl) supernatant + yeast	banana + + +, rose +	strawberry + +, tree fruits + +, raspberry ++, egriot cherry +++
(Bfp) pellet + yeast	fruit-salad, grassy + + + +	grassy + + + +
Skins after Sonication and Centrifugation:		
(Bsl) supernatant + yeast	aroma of Pinot + +, rose petals + + +	Ruché (floral) + +
(Bsp) pellet + SNM + yeast	typical aroma of Pinot + + + +	typical aroma of Ruché + + + +
Pulp and Seeds after Centrifugation:		
(Pt) uncentrifuged pulp and seeds + yeast	aroma of Pinot (floral) + +	Ruché (fruit) + +
(Pl) supernatant + yeast	aroma of Pinot (floral) + +	aroma of Ruché (fruit) + +
(Pp) pellet + SNM + yeast	banana +, rancid + +	exotic fruit + +, cheese + +
Control Samples:		
(Bg) crushed wool barriers (skins + pulp + seeds) + yeast	typical aroma of Pinot + + + +	typical aroma of Ruché + + + +
(Bt) wool skins + SNM + yeast	typical aroma of Pinot +	typical aroma of Ruché +
(Bf) fragmented skins + SNM + yeast	typical aroma of Pinot + + +	typical aroma of Ruché + + +
(C1) SNM + yeast without must components	yeasty odor of fermentation + +	yeasty odor of fermentation + +
(C2) SNM without yeast + whole skins	odor of soaked grass + + + +	odor of soaked grass + + + +
(C3) SNM without yeast + whole skins + sorbate	odor of soaked grass + + + +	odor of soaked grass + + + +
(C4) SNM without yeast + fragmented skins	odor of soaked grass + + + +	odor of soaked grass + + + +
(C5) SNM without yeast + fragmented skins + sorbate	odor of soaked grass + + + +	odor of soaked grass + + + +

[a] The increasing number of the + indicates the increasing odor intensity.

because they are randomly distributed by weight from a lot of berries grouped randomly in a heap. In fact, no organoleptic or compositional difference was seen between the two replicates of the same fermenting clone. Thus, this method appears as highly reproducible as possible within experimental error.

Furthermore, the comparison between a fermentation carried out in SNM/c with one carried out in must allows us to identify and narrow the choice to one genetic property, in other words, what the strain can produce with and without the complication of other plant components. In fact, in this comparative study the fermentation in SNM/c was carried out in the presence of only two ingredients generally present in must, glucose and ammonium nitrogen, along with all the essential vitamins and amino acids. On the other hand, the advantage of using a must fermentation is that it can be used to determine and evaluate the effects of a medium with a complex composition (typical of each grape variety), on the organoleptic properties of wine in each specific case. The proposed method has also the following advantages: (1) it permits a rapid screening of several yeast clones by using only laboratory scale materials (flasks, Pasteur micropipets, 100 g of berries/replicate) and a small amount of berries which can be fresh or frozen; (2) by being able to use berries stored at −20 °C, it allows a direct comparison between different vintages as a way of evaluating the influence of grape quality and climate; (3) SNM/c allows us to screen rapidly for strains that produce off-flavors.

Identifying the Source of Varietal Odors (Skin, Pulp, Seeds) Produced during a Yeast Fermentation (Experiment II). The data for exp II (Table 3) indicate that all samples containing must components (skins, pulp, seeds) and inoculated with yeast cells produced odors characteristic of the corresponding wine even if it was differently intense from samples to samples. All other samples without yeast addition or treated with potassium sorbate do not develop a characteristic wine odor even after incubating the entire experimental period (10 days). By contrast, samples without yeast and potassium sorbate, that spontaneously contaminated after 5 days showed an odor characteristic of wine from the sixth day of incubation. This accidental contamination is, however, a further confirmation that the formation of a wine odor only occurs if a berry skin and/or pulp come into contact with developing yeast cells.

Table 4. Olfaction Analysis of Fragmented Skins Incubated in SNM/m As Indicated

sample no.	substrate (skins)	yeast strain	incubation (days)	temp °C	aroma
1	Pinot Noir	none	2	25	vegetal
2	Ruche	none	2	25	vegetal
3	Pinot Noir	AM99	2	25	characteristic floral, fruity
4	Ruche	BR94	2	25	characteristic floral, fruity
5	Pinot Noir	AM99	7	50	caramelized
6	Ruche	BR94	7	50	cooked fruit, phenolic (chemical)
7	Pinot Noir	AM99	7	70	cooked fruit, phenolic (chemical)
8	Ruche	BR94	7	70	cooked fruit, phenolic (chemical)
9	Pinot Noir + ethanol	AM99	7	25	heady (ethanol)
10	Ruche + ethanol	BR94	7	25	heady (ethanol)

The skins homogenized in a kitchen Waring blender (fractions Bfl and Bfp), produced intense odors but with grassy and vegetable notes that particularly in Bfp obfuscated the typical odor of the wine in question.

Samples containing skins subjected to sonication (fractions Bsl and Bsp) revealed complex and floral odors that, although reminiscent of the wines in question, could not always be classified as typical. The effect of sonication on aroma formation may deserve a more in depth investigation.

In terms of clean intense floral and fruity odors, the best results were obtained with unrinsed skins that were split in two and then cut into coarse fragments (Bf).

Conditions Supporting the Expression of Primary Aromatic Markers (Experiment III). On the basis of the olfaction results listed in Table 3 (exp II), subsequent experiments were limited to the contribution of skins, leaving the contribution of pulp and seeds for a future study.

Table 5. Primary Aroma Content of Several Red Wines Determined by HS-SPME and GC-MS Analysis (Experiment V)

primary aroma compounds[a]	Ruchè	Malvasia	Brachetto	Pinot Gris	Pinot Noir	Nebbiolo	Merlot	Croatina	Cabernet
2,6,6-trimethyl-2-vinyl-4*H*-pyran	5.2	4.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
myrcene	3.3	3.3	4.8	0.0	0.0	0.0	0.0	0.0	0.0
β-pinene	4.6	4.5	2.4	0.0	0.0	3.2	0.0	<0.1	0.0
α-terpinene	3.3	5.3	3.6	0.0	0.0	0.0	0.0	0.0	0.0
limonene	3.6	10.5	12.8	2.8	2.8	52.8	2.9	1.6	2.2
cis-ocimene	3.3	0.0	4	0.0	0.0	0.0	0.0	0.0	0.0
trans-ocimene	5.9	7.3	2.8	0.0	0.0	0.0	0.0	0.0	0.0
terpinolene	7.3	16.4	6	0.0	0.0	0.0	0.0	0.0	0.0
2-octanone	33	32.2	0.0	1.7	<0.1	0.0	<0.1	2.6	3.1
linalyl ethyl ether	20.3	88.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6-methyl-5-hepten-2-one	9.9	16.3	6	1.7	2	2	1.8	<0.1	1.7
ethyl 2-hexenoate	7.9	22.3	7.2	15.4	6	6	6.5	1.6	2.2
cis-rose oxide	7.3	14.4	18	3.5	2.4	<0.1	3.6	<0.1	2.2
trans-rose oxide	3.3	3.3	8.8	0.0	0.0	0.0	0.0	0.0	0.0
cis-3-hexen-1-ol	6.6	<0.1	4	3.5	1.2	2	1.8	1.6	2.2
2-nonanone	4.9	3.3	4	0.0	0.0	0.0	9.3	2.6	1.7
trans-2-hexen-1-ol	8.9	0.0	6	3.5	<0.1	0.0	<0.1	0.0	0.0
not identified (59,87,136)	20.0	19.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
nerol oxide	13.5	17.5	16.8	0.0	0.0	0.0	0.0	0.0	0.0
trans-linalool oxide	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6-methyl-5-hepten-2-ol	9.9	11.1	4	2.4	0.0	0.0	0.0	1.6	0.0
a terpene	6.6	18	0.0	0.0	0.0	0.0	0.0	0.0	0.0
not identified	4.1	8.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
a terpene	21.4	79.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
vitispiran isomer 1	33.4	0.0	0.0	0.0	0.0	0.0	0.0	<0.1	0.0
vitispiran isomer 2	9.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2-methyl-4*H*-thiophen-3-one?	4.3	8.9	8	3.5	0.0	7.2	3.6	1.6	5.2
linalool	89.1	631	40.0	0.0	16	6	7.2	6.4	6.5
ethyl-3-methylthiopropanoate	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
not identified (43,138,148)	3.7	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
safranal?	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
citronellyl acetate	4.6	22.2	200	1.7	1.2	<0.1	<0.1	<0.1	0.0
cis-ocimenol	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
trans-ocimenol	9.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.3
a menthenol	8.6	2.7	4.8	0.0	0.0	0.0	0.0	0.0	0.0
a menthenol	9.9	5.2	8	0.0	0.0	0.0	0.0	0.0	0.0
α-terpineol	16.4	60.6	49	41	15	30	16	6.9	12
not identified (177, 192)	3.7	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3-(methylthio)propanol	4.3	5.1	2.4	2.1	8.8	10	8.3	1.6	8.7
neryl acetate	3.3	5.1	64	0.0	0.0	0.0	0.0	0.0	0.0
not identified (145,218)	2.7	<0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ethyl geranate	21.5	6.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
linalool oxide C	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
methyl phenyl acetate	9.9	0.0	2	<0.1	0.0	<0.1	0.0	0.0	3.5
methyl 2-hydroxybenzoate	10.6	3.9	2	<0.1	2	1.2	4	<0.1	0.0
geranyl acetate	3.3	5.1	30.0	0.0	0.0	0.0	0.0	0.0	0.0
citronellol	95.7	202	84	10.5	6	2.4	5.4	5.4	0.0
ethyl-2-hydroxybenzoate	3.3	0.0	0.0	0.0	0.0	<0.1	1.1	1	0.0
not identified (175,190)	4.0	1.5	0.0	0.0	<0.1	0.0	0.0	0.0	0.9
nerol	90.7	66.3	64	0.0	2	<0.1	<0.1	<0.1	0.0
a terpene	0.0	44.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-damascenone	13.5	34.1	8	3.5	8	8	7.2	2.6	8.7
geraniol	110.5	84.3	32	<0.1	4	0.0	0.0	1.6	6.5
4-ethyl-2-methoxyphenol	5.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4-ethylphenol	17.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ethyl 3-phenylpropionate	1.6	0.0	0.0	<0.1	<0.1	1.2	<0.1	<0.1	0.0
ethyl 3-OH-octanoate?	1.7	0.0	2	<0.1	<0.1	<0.1	<0.1	<0.1	0.0
methyl-α-hydroxy-3-phenyl-ketone?	7.3	0.0	0.0	<0.1	<0.1	<0.1	<0.1	<0.1	2.2
ethyl, 3-phenyl-2-OH-propionate	9.9	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	1.7
geranic acid	21.5	22.2	9.6	0.0	0.0	0.0	0.0	0.0	0.0
methyl vanillate	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ethyl vanillate	1.7	0.0	<0.1	0.0	0.0	0.0	0.0	0.0	0.0

[a] All data are expressed as area percent of the internal standard and are the average of three replicated analyses. <0.1 = signals at the level of background.

In a protocol similar to the one listed in Table 3, it was also observed in preliminary experiments that within the first 48 h, the intensity of the aroma released as the primary aromatic marker, increased as a function of yeast cell concentration up to an optimal concentration of $40-50 \times 10^6$ cells/mL, especially when added in their active growth phase (data not shown). Yeast cells in stationary phase were less responsive. SNM/m5g containing 50 mg/L ammonium (as NH_4SO_4) was sufficient to support 2−3 multiplications that significantly reduced the contribution of secondary aroma compounds. Other sugars substituted in SNM (lactose, melibiose, sorbose) also supported the expression of the primary aromas but with less intensity as compared to glucose (data not shown).

The effect of other experimental conditions were investigated (Table 4). Under normal conditions, samples 3 and 4, inoculated with AM99 and BR96 strains,

Table 6. Primary Aroma Content of Ruché as Compared to Brachetto, as Determined by HS-SPME and GC-MS Analysis (Experiment V)

	Brachetto		Ruché	
volatile compounds[a]	by skin−yeast contact method	by fermentation of whole grape must (skins + juice + seeds) + yeast	by skin−yeast contact method	by fermentation of whole grape must (skins + juice + seeds) + yeast
2,6,6-trimethyl-2-vinyl-4*H*-pyran	2.9	0.0	3.0	5.2
myrcene	5.6	4.8	15.0	3.3
β-pinene	2.2	2.4	7.0	4.6
α-terpinene	1.1	3.6	1.3	3.3
limonene	118.0	12.8	147.0	3.6
cis-ocimene	0.0	4.0	0.0	3.3
trans-ocimene	4.4	2.8	5.0	5.9
terpinolene	2.2	6.0	9.1	7.3
2-octanone	0.0	0.0	4.3	33.0
linalyl ethyl ether	0.0	0.0	2.7	20.3
6-methyl-5-hepten-2-one	17.4	6.0	58.0	9.9
ethyl 2-hexenoate	0.0	7.2	0.0	7.9
cis-rose oxide	16.0	18.0	14.1	7.3
trans-rose oxide	4.4	8.8	6.4	3.3
cis-3-hexen-1-ol	4.0	4.0	30.0	6.6
2-nonanone	0.0	4.0	26.1	4.9
trans-2-hexen-1-ol	3.3	6.0	20.0	8.9
not identified (59, 87, 136)	0.8	0.0	0.0	20.0
nerol oxide	8.3	16.8	3.0	13.5
linalool oxide B	0.0	0.0	0.0	3.3
6-methyl-5-hepten-2-ol	2.8	4.0	5.0	9.9
a terpene	0.0	0.0	0.0	6.6
a terpene	0.0	0.0	0.0	4.1
a terpene	1.3	0.0	0.0	21.4
vitispiran isomer 1	0.0	0.0	0.0	33.4
vitispiran isomer 2	0.0	0.0	0.0	9.8
2-methyl-4*H*-thiophen-3-one?	1.2	8.0	3.0	4.3
linalool	124.0	40.0	20.0	89.1
ethyl-3-methylthiopropanoate	0.0	0.0	0.0	1.8
hotrienol	1.7	4.0	0.0	3.3
safranal?	0.0	0.0	0.0	1.8
cis-ocimenol	0.0	0.0	0.0	3.3
citronellyl acetate	7.8	200.0	0.0	4.6
trans-ocimenol	0.0	0.0	0.0	9.9
a menthenol	2.2	4.8	7.1	8.6
a menthenol	5.6	8.0	9.6	9.9
α-terpineol	45.2	49.0	23.0	16.4
3-(methylthio)-1-propanol,	0.0	2.4	0.0	4.3
not identified (43,138,148)	0.0	0.0	0.0	3.7
not identified (145,218)	0.0	0.0	0.0	2.7
ethyl geranate	0.0	0.0	0.0	21.5
methyl phenylacetate	0.0	2.0	0.0	9.9
linalool oxide C	0.0	0.0	0.0	3.3
neryl acetate	2.2	64.0	0.0	3.3
methyl-2-hydroxybenzoate	2.2	2.0	3.0	10.6
geranyl acetate	4.4	30.0	1.6	3.3
citronellol	307.0	84.0	52.4	95.7
not identified	0.0	0.0	0.0	4.0
ethyl 2-hydroxybenzoate	0.0	0.0	2.0	3.3
nerol	195.0	64.0	156.7	90.7
not identified	0.0	0.0	0.0	3.7
β-damascenone	0.6	8.0	3.0	13.5
geraniol	104.0	32.0	228.0	110.5
benzene methanol	0.0	2.8	25.0	11.9
ethyl 3-phenylpropionate	0.0	0.0	0.0	1.6
ethyl-3-hydroxyoctanoate?	0.0	0.0	0.0	1.7
methyl-α-hydroxy-3-phenyl-ketone?	0.0	0.0	0.0	7.3
ethyl 3-phenyl-2-hydroxypropionate	0.0	0.0	0.0	9.9
geranic acid	41.3	22.2	6.0	21.5
methyl vanillate	0.0	0.0	0.0	1.7
ethyl vanillate	0.0	0.0	0.0	1.7

[a] All data are expressed as area percent on the internal standard pick area and are the average of three replicated analysis.

respectively, demonstrated the floral and fruity perfumes characteristic of the two wines (Table 4). The characteristic odors were detected within 24 h and increased in intensity during the subsequent 24 h. The uninoculated controls (samples 1 and 2) only expressed the initial vegetal odor of soaked skins (Table 4).

In samples 9 and 10, the ethanol addition does not produce any significant odor.

At 50 or 70 °C, caramelized or cooked fruit or phenolic (chemical) odors were expressed (Table 4).

At the end of the experiment, the samples to which yeast was added produced 2−3% ethanol and the yeast

Table 7. Olfaction Analysis of Different Grape Varieties by the Rapid Method for Primary Aroma Expression Using Yeast Contact of Fragmented Skins (Experiment VI)[a]

vine variety	olfaction description	odor intensity	degree of correspondence with the wine control sample
(1) white berry			
Arneis	fruity perfumes (apricot); herbaceous odor	++++	+++
Cortese	fruity perfumes (apricot); jam; herbaceous odor	+++	+++
Erbaluce	typical aroma of the correspondent wine; wood, tobacco	++++	+++
Favorita	typical aroma of the correspondent wine; soft spices aroma	++++	+++
Moscato	typical aroma of the Moscato wine (linalool)	++++	+++
2) red berry			
Barbera	caramel; spices aroma (licorice, pepper)	++	++
Brachetto	typical aroma of the correspondent wine; floral notes (rose) and woody odor	++++	++++
Cabernet S.	high spices aroma; dry fruit; wood fruits (berry)	+++	+++
Croatina	generic wine aroma; wood fruits; herbaceous; pepper	++	+
Dolcetto	typical aroma of the correspondent wine; floral and fruity odors	++++	+++
Grignolino	typical aroma of the correspondent wine; woody aroma; marmalade	+++	++++
Merlot	delicate wine odor; fruity (fresh grape, pear)	++	++
Nebbiolo	soft and sweet perfumes; fatty and phenolic odors	+++	++++
Pinot Gris	typical aroma of the correspondent wine	+++	+++
Pinot Noir	typical aroma of the correspondent wine	+++	++++
Ruchè	typical aroma of the correspondent wine; floral (rose); spices (cinnamon)	++++	++++

[a] The increasing number of the + indicates the increasing odor intensity.

population increased to $90-120 \times 10^6$ cells/mL, equivalent to 2 or 3 rounds of multiplication.

The presence of oxygen did not appear to be an important factor since mutant HRO^- of AM 99 supported the release of Pinot Noir primary markers with the same intensity as did the wild-type HRO+ strain (data not shown).

Acidic Hydrolysis (Experiment IV). Since vinification occurs under acid conditions, it was of interest to determine the contribution of prolonged exposure to acid conditions on the release of aroma from grape skins.

Whether the skins were cut into coarse fragments or homogenized in a kitchen blender, the results (data not shown) indicated that a nonvinous vegetal odor persisted in all samples for the first seven days of exposure. From the eighth day, the samples that were incubated aerobically (1a) and anaerobically (1b) at pH 1.1 developed a sweet smelling aroma very characteristic of dry hay or mint, which increased in intensity with time. In samples incubated aerobically at pH 1.1 (1a) and samples incubated anaerobically at pH 3.2 (2b) (with or without potassium sorbate and SO_2), the initial vegetal odor persisted to the end of the experiment.

Since typical Ruché and Pinot Noir aromas did not develop, these results strongly suggest that under normal technological conditions of vinification (incubation temperatures between 15 and 28 °C and at pH between 2.9 and 4.0), aroma production does not occur by acid hydrolysis of covalently bound precursors in the media.

Aroma Profile of Ruché Wine in Comparison with Other Red Wines (Experiment V). Aroma analysis of the wine produced by microvinification with Ruché whole must indicated that the HS-SPME and GC-MS chromatographic profiles of Ruché, Brachetto, and Malvasia wines, were similar, but differed qualitatively and quantitatively from the other wine varieties examined (Table 5). The major constituents that contributed to the aroma in these three wines were identified as terpenols and esters of linalool, citronellol, nerol, and geraniol (Table 5). In addition, a comparison of the aroma profiles of Ruché and Brachetto produced by the yeast-skin contact method with the aroma profile produced by fermentation of whole grape must (Table 6) revealed significant qualitative and quantitative differences between the two methods for many aroma compounds. Specifically, Ruché shows a larger number of volatile compounds in the whole grape must sample than in the skin-yeast contact sample, while Brachetto shows lower qualitative differences between the two methods. In addition, some primary aroma compounds are found in both Brachetto and Ruché (limonene, 6-methyl-5-hepten-2-ol, nerol and geraniol) while others such as *cis*-3-hexen-1-ol, 2-nonanone and *trans*-2-hexen-1-ol are found in Ruché and in higher concentrations in the skin−yeast contact sample. Interestingly, the concentrations of linalool, citronellol, and geranic acid are inverted in the two sampling methods used for Brachetto and Ruché. As expected, acetate esters appear in higher concentrations in the whole grape must sample than in the skin−yeast contact sample. These results suggested that the rapid and inexpensive laboratory "yeast−skin contact method" was worthy of further investigation.

Wine Odor Obtained by Skin−Yeast Contact (Experiment VI). Skins from the grape varieties listed in Table 7 were isolated, fragmented, and incubated as described under exp VI. Both the aromatic and nonaromatic (red or white) wines listed in Table 7 show the formation of wine odor typical of the grapes tested. In particular, distinctive aromatic notes were found associated with Arneis, Brachetto, Cortese, Moscato, and Ruché. By contrast, the control samples without added yeast did not support odor formation.

However, the odors found in the skin−yeast contact samples were never identical to those of the corresponding wine control (Table 6). A likely explanation is that the reduced fermentation activity by yeast in the skin−yeast contact method results in a smaller production of volatile compounds and thus less volatiles in the skin−yeast contact samples as compared to wine control samples. This observation suggests an important advantage since only the fragrance coming from the

varietal precursors in the skins are produced without being obscured by larger amounts of other volatile yeast compounds, contrary to what normally occurs in fermentation with whole must. The skin-contact method appears to be an effective rapid screen during yeast and/or vine clonal selection for obtaining within a short period of time (48 h) a very good approximation of the typical wine odors that is normally expressed in technological fermentation from any grape variety.

Wine Odor and Primary Aroma Compounds Obtained by Varietal Precursors−Yeast Contact (Experiment VII). All three methods described under exp VII for extracting aroma precursors from Ruché skins manifested the odor of Ruché wine but in different intensities and complexities. After yeast contact, the greatest intensity was found in samples extracted with water-saturated butanol, whereas the methanol extract displayed grassy and fruity odors, while the buffer Bm extract showed primarily floral odors (Table 8).

Methylene chloride extraction of the culture supernatants revealed by GC-MS analysis that butanol was the better solvent for extracting primary aroma precursors from the plant material, not only for identification but for quantification as well (Table 8). Geranic acid and geraniol represented the largest concentration in the butanol extract, while all the other aroma markers were found to be present in amounts not very different from the average amount normally found in other wines. Furthermore, no new aroma markers were detected in Ruché wine.

In addition, the content of the aqueous butanol extracts revealed a greater complexity by GC-MS spectrometry (Table 8) than the microextract obtained by SPME (Tables 5 and 6). The difference, presumably, was because aqueous butanol extract contained more of the less volatile compounds.

In the GC-MS chromatogram, geraniol appears to be quantitatively the more prominent terpenol, and the *trans*-2,6-dimethyl-3,7-octadiene-2,6-diol and the *trans*-3,7-dimethyl-2-octen-1,7-diol, the more significant diols.

In samples in which yeast produced only a maximum of 0.2% alcohol, typical wine odors could not be detected, suggesting lack of sugar fermentation and, thus, of yeast secondary compound production (e.g., esters). By contrast, in the absence of added yeast, neither alcohol nor wine odor was produced, although the skins contained a large amount of primary aroma precursors (Table 9). Furthermore, contrary to what was expected, the quantity of aroma precursors found in some samples without added yeast (controls) (Table 9), was greater than the amount found in samples to which yeast was added. This anomalous response occurred with geraniol, 4-vinylguaiacol, hydroxycitronellol, 3,7-dimethyl-2-octen-1,7-diol, geranic acid, and vanillyl acetone. However, some differences could be explained by the precursor extraction technique used (e.g., for 2-H-pyran,2-ethenyltetrahydro-2,6,6-trimethyl-, terpinolene, hotrienol, etc.) (Table 9).

Such anomalous results beg the question: what factor(s) present in control samples (no added yeast) allows the release of primary aromas in some samples but not in others? After excluding acid hydrolysis as the source of primary aroma, the activity of specific "aromatic" grape enzymes can be hypothesized as well as the absence and/or inactivation of yeast enzymes. It is unlikely that grape enzymes could have survived the extraction procedure except when aqueous buffer or SNM was used. However, the absence of yeast enzyme activity could suggest unknown compounds in the

Table 8. Effect of the Solvent in the Extraction Efficacy of Primary Precursors[a]

primary aroma compounds (μg/L)	aromatic precursors solvent used		
	butanol−water	methanol	buffer Bm
2-ethenyltetrahydro-2,6,6-trimethyl-2H-pyran	165.7	30.6	96.0
myrcene	33.8	0.0	0.2
β-pinene	226.4	13.8	23.3
α-terpinene	44.6	0.0	0.0
limonene	145.4	10.4	28.6
cis-ocimene	80.4	3.8	16.3
γ-terpinene	23.6	2.8	0.0
trans-ocimene	146.4	8.6	17.4
terpinolene	38.5	2.8	6.4
6-methyl-5-hepten-2-one	56.4	12.6	26.7
1,3-oxathiane?	24.8	28.8	26.6
trans-linalool oxide	76.7	14.4	18.2
cis-linalool oxide	259.4	24.0	34.4
vitispirane isomer 1	10.2	7.2	2.6
vitispirane isomer 2	13.8	5.8	2.6
linalool	31.4	6.4	9.2
hotrienol	179.1	96.0	123.3
myrcenol	115.4	28.8	39.9
cis-ocimenol	62.9	20.2	23.6
trans-ocimenol	103.8	26.0	36.8
α-terpineol	70.0	5.8	5.6
trans-pyran-linalool oxide	15.1	5.8	25.5
cis-pyran-linalool oxide	38.7	2.8	5.6
citronellol	44.6	0.0	15.8
geranyl acetate	39.3	2.8	7.8
nerol	90.2	15.0	104.2
geraniol	516.9	17.2	434.0
benzene methanol	420.0	427.4	1042.6
2,6-dimethyl-3,7-octadiene-2,6-diol	1044.3	104.0	293.4
2,6-dimethyl-7-octen-2,6-diol	0.0	11.8	47.2
4-vinylguaiacol	748.7	13.0	5.7
hydroxycitronellol	0.0	43.8	0.2
methyl-β-hydroxy-3-phenyl-ketone?	20.0	11.6	0.0
methyl-α-hydroxy-3-phenyl-ketone?	25.0	11.0	0.0
ethyl-3-phenyl-2-hydroxy-propionate	17.0	0.0	0.0
dihydroactinidiolide	36.4	22.4	56.8
trans-3,7-dimethyl-2-octen-1,7-diol	891.9	371.6	1129.1
geranic acid	6013.8	1577.0	2579.2
benzoic acid	172.6	53.8	197.7
not identified (111,121)	1756.2	181.8	0.0
p-menthen-7,8-diol	0.0	0.0	39.8
3-hydroxy-β-damascone	0.0	14.4	0.0
4-hydroxy-3-methoxy-benzaldehyde	0.0	14.4	0.0
benzenacetic acid	188.7	20.2	122.9
methyl vanillate	857.4	494.0	445.8
3,5,5-trimethyl-4-(3OH-butenyl)-2-cyclohexenone?	157.0	28.8	46.9
ethyl vanillate	162.5	34.6	15.6
acetovanillone	212.7	51.8	200.5
3-hydroxy-β-ionone	74.1	86.4	171.2
vanillyl acetone, isomer	176.9	46.6	143.2
ethoxy-4-methoxy benzaldehyde	224.5	57.6	159.4
β-ionone-5,6-epoxide	493.1	39.2	60.9
vanillyl acetone	83.1	25.0	88.5
4-hydroxy-3-methoxy acetophenone	45.0	28.8	0.0
methyl vanillyl ether?	245.1	5.8	125.7

[a] Data refer to primary aroma compounds found in Ruché skin precursor extracts obtained with the solvents listed and after contact with yeast (exp VII)

Table 9. Primary Aroma Compounds Found in Ruché Skin Extracts (1 and 2) with or without Yeast Treatment and Identified by Liquid−Liquid Methylene Chloride Extraction and GC-MS Analysis (Experiment VII) in Comparison with Ruché Wine and a Control Sample without Any Previous Skin Precursor Extraction[a]

			extraction technique of aroma precursors from Ruché skins					
			control samples (whole skins + SNM/m)		by butanol−water and C18 + SNM/m5g		by buffer Bm and C18 + SNM/m5g	
RT	primary aroma compounds (μg/L)	wine Ruchè	+ yeast	no yeast	+ yeast	no yeast	+ yeast	no yeast
10,19	2-H-pyran, 2-ethenyltetrahydro-2,6,6-trimethyl-	823	122.2	100.0	165.7	204.3	96.0	91.5
12,53	myrcene	53	0.0	0.0	33.8	40.9	0.2	0.0
13,26	β-pinene	91	39.1	28.0	226.4	108.2	23.3	26.5
13,46	α-terpinene	41	0.0	0.0	44.6	30.5	0.2	0.0
14,50	limonene	171	28.6	38.6	145.4	65.9	28.6	22.9
17,23	*cis*-ocimene	76	116.3	21.7	80.4	40.2	16.3	14.9
17,21	γ-terpinene	50	52.2	29.2	23.6	33.5	0.2	0.0
17,94	*trans*-ocimene	121	95.2	34.7	146.4	68.3	17.4	18.1
19,40	terpinolene	221	28.6	44.6	38.5	27.4	6.4	8.0
23,01	6-methyl-5-hepten-2-one	107	15.2	21.7	56.4	40.2	26.7	34.0
24,11	1,3-oxathiane?	56	359.6	48.2	24.8	8.2	26.6	0.2
29,28	*trans*-linalool oxide	151	32	9	76.7	55.5	18.2	12.4
31,08	*cis*-linalool oxide	354	30	10	259.4	74	34.4	19.9
33,95	vitispiran isomer 1	26	50.1	43.4	10.2	7.6	2.6	0.2
34,13	vitispiran isomer 2	23	62.5	54.2	13.8	10.7	2.6	0.2
36,34	linalool	212	139.6	22.4	31.4	25.6	9.2	14.1
40,04	hotrienol	2385	112.1	200.0	179.1	124.7	123.3	49.5
40,23	myrcenol	227	61.3	54.2	115.4	116.5	39.9	23.9
42,69	*cis*-ocimenol	303	45.9	34.5	62.9	62.5	23.6	15.7
44,09	*trans*-ocimenol	424	62.8	27.0	103.8	166.2	36.8	30.9
44,81	α-terpineol	136	22.4	6.5	70.0	29.6	5.6	10.3
47,83	*trans*-pyran-linalool oxide	92	37.2	25.1	15.1	39.0	25.5	36.7
50,20	*cis*-pyran-linalool oxide	64	8.5	5.8	38.7	19.2	5.6	6.1
50,78	citronellol	378	47.4	10.4	44.6	0.0	15.8	24.3
52,34	geranyl acetate	49	57.1	12.1	39.3	30.5	7.8	13.4
53,79	nerol	106	88.0	75.2	90.2	70.1	104.2	206.7
59,49	geraniol	360	272.8	248.7	516.9	646.1	434.0	752.5
61,50	benzene methanol	651	788.5	841.1	420.0	419.8	1043	1022
69,23	*trans*-2,6-dimethyl-3,7-octadiene-2,6-diol	156	39.8	57.6	1044	416.8	293.4	270.1
71,61	2,6-dimethyl-7-octen-2,6-diol	361	24.5	19.3	0.0	0.0	47.2	38.2
83,85	4-vinyl-guaiacol	112	27.1	49.4	748.7	170.4	5.7	7.6
85,09	hydroxycitronellol	516	36.0	22.7	0.0	0.0	0.2	170.9
86,61	methyl-β-hydroxy-3-phenyl-ketone?	203	195.2	0.0	20.3	0.0	0.0	0.0
87,43	methyl-α-hydroxy-3-phenyl-ketone?	694	283.4	0.0	25.1	0.0	0.0	0.0
87,65	ethyl-3-phenyl-2-hydroxy propionate	1851	152.3	0.0	17.4	0.0	0.0	0.0
89,94	dihydro-actinidiolide	50	26.6	11.6	36.4	0.0	56.8	73.2
90,26	*trans*-3,7-dimethyl-2-octen-1,7-diol	1147	179.4	358.6	891.9	1122	1129	896.4
91,40	geranic acid	2760	1464.0	714.1	6014	7915	2579	3333
94,58	benzoic acid	0.0	165.2	31.3	172.6	102.4	197.7	150.5
96,68	not identified (111,121)	0.0	300.8	260.3	1756	1110	0.2	0.0
97,71	*p*-menthen-7,8-diol	109	67.5	23.4	0.0	0.0	39.8	0.0
98,06	3-hydroxy-β-damascone	182	185.9	147.0	0.0	0.0	0.0	0.0
98,71	4-hydroxy-3-methoxy-benzaldehyde	39	105.1	81.9	0.0	0.0	0.2	156.6
98,69	benzenacetic acid	500	106.6	0.0	188.7	775.4	122.9	151.3
100,00	methyl vanillate	757	452.2	348.7	857.4	148.8	445.8	522.6
100,71	2-cyclohexenone-3,5,5-trimethyl-4-(3OH-butenyl)?	94	86.7	59.8	157.0	97.3	46.9	20.6
100,77	ethyl vanillate	1453	149.1	98.3	162.5	171.4	15.6	74.9
100,88	acetovanillone	800	152.3	85.6	212.7	575.0	200.5	215.6
102,09	3-hydroxy-β-ionone	50	86.5	69.9	74.1	285.4	171.2	139.6
102,53	vanillyl acetone, isomer	0.0	92.2	68.7	176.9	461.6	143.2	100.3
102,79	ethoxy-4-methoxy benzaldehyde	189	97.3	48.0	224.5	647.6	159.4	64.9
103,54	β-ionone-5,6-epoxide	15	31.7	47.2	493.1	598.5	60.9	74.5
104,76	2-hydroxy-β-ionone	50	50.8	23.1	0.0	0.0	0.2	0.0
106,00	vanillyl acetone	182	117.2	118.6	83.1	132.6	88.5	120.3
106,81	4-hydroxy-3-methoxy acetophenone	60	109.3	49.4	45	0.0	0.2	0.0
108,08	methyl vanillyl ether?	368	129.9	96.4	245.1	230.2	125.7	432.4

[a] RT = retention time.

extract setting up competing reactions or unfavorable enzyme conditions. Alternatively, the yeast strains used may lack other enzymes such as pectolytic, proteolytic, cellulolytic, etc., that prepare the precursor substrates for the "aromatic enzymes" (i.e., glycosidases, cysteine conjugate β-lyase). In addition, it is conceivable that yeast could have metabolized the aroma precursor completely.

Furthermore, if we also consider the results of the GC-olfactometry analysis performed on Ruché wines, on "skin−yeast contact" (Table 4) and "precursor−yeast contact" (Table 9) samples, we have to admit that only markers and odors well-known and common to many wines were found. For instance, in the liquid−liquid methylene chloride extraction procedure of Ruché wine (that was the most complex olfactometry spectra ob-

tained), the following odor descriptors were found (in order of retention times on the chromatogram): licorice, chocolate, crème fruity, butter, strawberry, wood fruits, cooked fruit, leaves, spices, coffee, plastic, pyrethrum, acid apple, filbert, grassy odor, burnt plastic, flowery, mushroom, sulfur compounds, cherry, mint, vinegar, excrement, garlic, sweaty feet, cheese, anise, fennel, burnt bread crust, rose, capers, olives, leather. In any case, if these single odors cannot explain the typical and complex fragrance encountered in Ruché wines, we cannot exclude that the complexity could be explained if the single odors were allowed to interact in quantitative ratios identical to the ones found in wine.

Finally, we have to accede to the analytical limits of the system and the possible introduction of artifacts in the olfactometry analysis, especially when the identity of the compounds as the source of the odor cannot be confirmed with authentic pure compounds by GC-olfactometry, or artifacts introduced by using different techniques of injection (on column, cold purge and trap) and sniffing. The possibility that high-temperature injection can result in false odors or lack of odors cannot be denied. Furthermore, in some cases, the olfaction analysis performed on the spent aqueous fractions from the liquid−liquid methylene chloride extraction, revealed a residual pungent odor of acid, rancid fat, fruitwood, cacao, crushed apples, jam, cooked plum, and chestnut puree. Even if these residual odors could be artifactual consequences of the extraction technique and solvent evaporation, they should still be investigated to achieve a better understanding of the fragrance characteristic of Ruché wine. Eventual losses of very volatile unknown compounds during concentration under vacuum should be also taken into consideration. However, headspace SPME technique without any concentration under vacuum was also used (Tables 5 and 6).

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