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An Interplay between a Face-Centred Composite Experimental Design and Solid-Phase Microextraction for Wine Aroma GC/MS Analysis

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Abstract: For oenological products, most of the intrinsic and extrinsic drivers of perceived quality are associated with specific aromatic profiles. Aromatic diversity has been recognized as a central element in perceived quality as it is able to transmit the complex interactions between grape variety, geographical characteristics, and viticultural and winemaking practices, including the fermentative process. A comprehensive characterization of flavour compounds by headspace solid-phase microextraction (HS-SPME) and gas chromatography coupled to mass spectrometric analysis is often needed in order to ascertain the quality of wine. HS-SPME requires a proper optimization that can be achieved through an adequate experimental design. Here, a HS-SPME/GC-MS based method was developed to investigate the volatile compounds of wine samples obtained by laboratory-scale fermentations. This was performed by inoculating a commercial *Saccharomyces cerevisiae* strain, which is used both as single starter and as mixed starter, with an indigenous *Hanseniaspora osmophila* strain. The experimental conditions of HS-SPME (extraction temperature and time) were optimized by applying a face-centred composite experimental design. Up to 95% of the total variance was explained by the proposed model. The optimized method allowed us to confirm the usefulness of combining the inoculation of grapes with selected yeast strains in co-culture situations in order to improve the wine bouquet.

Keywords: experimental design; HS-SPME; wine aroma; *Saccharomyces cerevisiae*; *Hanseniaspora osmophila*

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1. Introduction

The overall aroma of wine is determined by a complex pool of volatile compounds, many of which are present at levels above their olfactory thresholds, and the aroma is one of the most striking features out of those that determine the consumers' choice. In order to address the consumers' preferences, wineries continuously adapt their business strategies and production practices [1]. Among the factors of the winemaking processes contributing to the sensorial complexity of the oenological products, i.e., fermentation strategies, grape varieties, geographical origin and wine aging [2], the selection of a suitable starter culture is a useful biotechnological tool with which to increase the number of odorant compounds responsible for the wine organoleptic characteristics. The use of

controlled multi-starter fermentations based on non-*Saccharomyces* and *Saccharomyces* strains is currently considered as a valid tool for enhancing wine complexity, even though the antagonistic interactions among these kind of yeasts are complex and still not entirely clear.

Other advantages associated with the multi-starter fermentation in winemaking are the possibility of increasing the total acidity, of reducing the ethanol content and of controlling the spoilage of the microflora in the wine [3]. The successful evolution of mixed starter fermentations is correlated with the active participation of non-*Saccharomyces* strains in the fermentative process, as these yeasts are usually less competitive than those of *S. cerevisiae*. Among the practices which are useful in increasing the persistence of non-*Saccharomyces* strains during the fermentation process, the use of immobilized cells rather than free cells gave promising results. Microbial cells can be immobilized through adsorption, covalent binding, gel entrapment into inert natural polymers, such as alginate, agarose, chitosan and pectin; these techniques defend the microbial cells from stress factors emerging during the fermentative process, preserving the metabolic activities correlated to the production of aroma compounds [4]. The effect of different starter formulations on wine bouquet can be studied through an untargeted profiling of the volatile organic compounds (VOCs) which occur in the samples after their preliminary extraction via gas chromatography coupled to mass spectrometry (GC-MS). Mass spectrometry coupled to chromatographic separation techniques have become the techniques of choice for the identification of unknown compounds in several fields, including the field of food chemistry [5–8]. Although some researchers have realized a VOC extraction by solid-phase extraction (SPE) or liquid–liquid extraction (LLE) using dichloromethane or ether/pentane as solvent [9–12], the best option for obtaining a complete volatile fraction is the headspace solid-phase microextraction (HS-SPME). HS-SPME is associated with a low risk of overvaluing odorants which have been poorly transferred to the headspace. Moreover, the volatile compounds are not lost during the evaporation, as in LLE, and the extracts are completely free from non-volatile material [13]. The HS-SPME conditions, such as fibre type, extraction temperature and time, widely influence the efficiency of the extraction and, above all, the vapor pressure and equilibrium of the aroma compounds in the headspace of the sample. The selection of the most appropriate SPME fibre depends on the target compounds and the studied matrix. In general, 100 μm of PDMS fibre allows for the adsorption of a higher number of analytes for wine samples and will allow fora higher degree of reproducibility and chemical and mechanical robustness than other compounds, such as the triphasic ones [14–17]. In detail, Bianco et al. [17] achieved a better precision with the non-polar PDMS phase compared to **the** DVB/CAR/PDMS phase. On the other hand, Sagratini et al. [14] found that PDMS fibre is particularly selective for esters which positively contribute to the quality of wine products, such as ethyl octanoate, ethyl-9-decenoate and ethyl decanoate. In addition to fibre type selection, extraction time and temperature optimization, sample saturation with a salting out agent has also been proven to enhance the extraction efficiency. This occurs due to the increase in the ionic strength of the solution and the decreased solubility of the apolar analytes in the solution [18].

Most of the practical work involving SPME optimization uses strategies based on systematic studies of one variable at a time (OVAT), during which all the variables affecting the SPME efficiency (i.e., fibre coating, extraction time and temperature, ionic strength and sample volume) are maintained at a constant level during test runs, except for the one being studied. However, the use of the classical univariate approach could result in incorrect optimizations since it does not consider the interactions between different factors, assuming instead that all the variables are independent and the effect, at a given set condition, is also the same if the remaining variables are changed [19]. In order to avoid erroneous run tests, an optimization planned carried out according to an experimental design (DoE) is the best option. Indeed, in DoE all variables are studied at the same time, enabling a reduction in the number of experiments with a complete exploration of the experimental domain [20,21]. Thus, it is a very suitable means with which to investigate most of the

variables involved in the HS-SPME process. Anyway, it should be considered that the quality of the results of an experimental design depends on the distribution of experiments in the experimental domain. When DoE does not allow the resolution of the problem, a redefinition of the experimental domain or a change in the postulated model could be needed [22].

Here, a face-centred composite experimental design has been developed, taking advantage of information gained from previously acquired experimental data. This design was used to optimize the headspace solid-phase microextraction of the volatile compounds occurring in experimental wine samples. The samples were obtained by laboratory-scale fermentations performed in Aglianico del Vulture grape must, one of the most diffuse grape varieties in the Basilicata region of Southern Italy [23,24]. The grape must was inoculated with a commercial *Saccharomyces cerevisiae* strain, used both as a single starter and as a mixed starter in combination with an indigenous *Hanseniaspora osmophila* strain, as both free and immobilized cells.

The optimized SPME-GC/MS method was used to ascertain the aromatic compounds of the experimental wines obtained by different starter cultures.

2. Materials and Methods

2.1. Chemicals and Reagents

Glacial acetic acid (≥99.99%), sodium chloride, absolute ethanol (EtOH, 99.8%), alginic acid sodium salt and the analytical standards of nerolidol (98%) and 1-butanol, 3-methyl-acetate (≥99%) were purchased from Sigma Aldrich (Milan, Italy). The analytical standard of 2-phenylethyl alcohol (98%) was obtained from Carlo Erba (Milan, Italy). A Milli-Q RG system (Millipore, Bedford, MS, USA) was used to produce ultrapure water. A standard mixture of acetic acid (3 mg/mL), 1-butanol, 3-methyl-acetate (3 mg/mL), phenylethyl alcohol (17 mg/mL) and nerolidol (0.070 mg/mL) was freshly prepared in EtOH/H_2O (13% *v*/*v*) and was used as model to optimize the main parameters affecting the headspace solid-phase microextraction efficiency for subsequent wine flavour analyses.

2.2. Wine Samples

Three wine samples were analyzed in this work which belonged to the same year and batch, namely: W1 and W2, i.e., wine samples obtained by fermentation with *S. cerevisiae* in co-culture with free and immobilized cells of the *H. osmophila* strain, respectively; wine sample C, i.e., the control obtained by fermentation with free cells of *S. cerevisiae* alone. The wild strain of *H. osmophila* ND1 belonged to the Yeast Collection of the University of Basilicata (UBYC), while the commercial strain of *S. cerevisiae* EC1118 was purchased from Lallemand Inc. (Toulose, France). The fermentations were performed in 2 L of pasteurized natural grape must (Aglianico del Vulture variety) at 26 °C; they were monitored by determination of weight loss due to CO_2 production during sugar fermentation. Grape must was pasteurized at 90 °C for 20 min in order to avoid the growth of undesirable microorganisms [25]. The fermentation process was stopped when weight and °Brix reductions were constant for three consecutive days [26]. Around 20 days were taken by each starter to complete the process. After headspace solid-phase microextraction optimization, all the wine samples were analyzed in triplicate by HS-SPME/GC-MS.

2.3. Optimization of Headspace Solid-Phase Microextraction

For the headspace solid-phase microextraction optimization, 3 mL of standard mixture were placed into 5 mL vials and saturated with 0.6 g of sodium chloride, according to the recommendations of a previously reported method [17], in order to obtain a liquid-phase-to-headspace-volume ratio, 1/β, of 0.6. The vials were sealed with polytetrafluoroethylene/silicone septum caps and equilibrated in a reacti Therm heating stirring module (Thermo Scientific, Pierce Protein Research Products, Rockford, IL, USA) for 10 min at the set temperature (as reported in Table 1) before the headspace sampling was performed.

After the sample/headspace equilibration period, the septum of vials was pierced with the needle containing the fibre retracted. Then, the fibre was exposed to the headspace for the fixed time and temperature. In total, 100 μm PDMS fibre was used. The extraction time and temperature were optimized by DoE.

Table 1. Levels of the variables (extraction time and extraction temperature) explored in the face-centred composite experimental design experiments (DoE).

Variable	Coded Levels		
	(−1.0)	**(0.0)**	**(+1.0)**
Extraction time (min)	10	20	30
Extraction temperature (°C)	40	50	60

2.4. Experimental Design

A face-centred composite experimental design was applied in order to perform an optimization of the HS-SPME. The variables investigated, i.e., extraction temperature and extraction time, were evaluated at three different levels. The independent variables and their related codes and levels are displayed in Table 1. The response evaluated during all experiments was the total sum of peak areas of the standard mixture, and relevant data were obtained in the GC-MS analysis. Three replicates were performed at the central point in order to quantify that the experimental error and response surface methodology were realized to choose the optimum values of temperature and extraction time for the GC-MS analysis of the aroma compounds of the three wine samples. The statistical experimental design and optimization calculations were performed using the R-based software CAT [27]. Regression analysis for the experiment data was performed and the results were fitted into a second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{k} \boldsymbol{\beta}_i \boldsymbol{x}_i + \sum_{i=1, i<j}^{k=1} \sum_{j=2}^{k} \boldsymbol{\beta}_{ij} \boldsymbol{x}_j + \sum_{i=2}^{k} \boldsymbol{\beta}_{ii} \boldsymbol{x}_{ii}^2$$

where β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients; x_i and x_j are the coded levels of independent variables affecting the dependent response Y; and k is the number of parameters.

2.5. GC-MS Analysis

The extracted analytes were desorbed into the injection port of the GC–MS system for 15 min at 260 °C. GC-MS analyses were performed on an Agilent Hewlett Packard 6890 plus gas chromatograph. This equipment was fitted with a split/splitless injector and an Agilent 5975 mass spectrometer (MS) detector, equipped in turn with a single quadrupole analyzer. The chromatographic separation was carried out on a HP-5MS capillary column 30 m × 0.25 mm i.d. × 0.25 μm film thickness (Agilent Technologies, Santa Clara, CA, USA). A deactivated glass liner 105 × 8.0 × 0.75 mm (Supelco, Milan, Italy) was installed in the injector which was used in splitless mode. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was adapted from Sagratini et al. [14] and programmed as follows: from 35 °C (hold for 0.5 min) to 50 °C (3 °C/min, hold time 2 min) and up to 250 °C (8 °C/min, hold time 2 min). Finally, a temperature of 270 °C was reached at 8 °C/min and held for 2 min. The electron ionization (EI) mode was used for the MS analysis, with an electron energy of 70 eV. The MS acquisition was performed in full-scan mode in the range m/z 30–400. The source temperature and the transfer line temperature were set, respectively, at 230 °C and 300 °C. Enhanced data analysis (Agilent Software, Santa Clara, CA, USA) was performed for the chromatographic data acquisition and processing, while Sigma Plot 11.0 (Systat Software, London, UK) was used for data elaboration and plotting.

3. Results and Discussion

3.1. HS-SPME Optimization by Experimental Design

The flavour profile, alongside the sugar–acid balance, is among the most important parameters contributing to the overall quality of wine products [28,29]. The qualitative determination of wine volatile compounds allows researchers to define an aromatic fingerprint. This is characteristic of a specific oenological product and is the result of several factors, including grape variety, fermentative and post-fermentative processes. The volatile fraction fingerprinting, performed by solid-phase microextraction, combined with direct analysis via gas chromatography coupled to mass spectrometry needs a preliminary optimization of the experimental parameters. A standard solution containing acetic acid, 1-butanol 3-methyl-acetate, 2-phenylethanol and nerolidol in EtOH/H_2O was employed for HS-SPME optimization. Since the analytical standards used belonged to the main four classes of volatile compounds occurring in the wine, namely acids, esters, alcohols and terpenes, they can be properly used as a model to mimic the effects of the studied matrix [30]. A 100 μm PDMS fibre was used for the adsorption of a higher number of analytes for wine samples.

The optimization of the extraction time and temperature was conducted by running experiments according to a face-centred composite design to select the best conditions of wine aroma compounds extraction with the lowest number of experiments. The extraction time was varied between 10 and 30 min, while the temperature ranged from 40 °C to 60 °C, as higher temperatures could change the composition of the wine sample by producing artifacts [15]. In total, 9 runs were carried out in triplicate to study the influence of the selected factors and their interactions on the HS-SPME. The results obtained for the responses, i.e., the total sum of peak areas of the standard mixture, are reported in Table 2.

Table 2. Values of the response obtained at the variation levels chosen for the variables selected for HS-SPME optimization by experimental design, i.e., extraction temperature and extraction time.

Experiment	Variables		Response
	Temperature (°C) (°C)	Time (min) (min)	Total Area
1	40	10	3.26×10^{8}
2	40	20	4.40×10^{9}
3	40	30	2.79×10^{8}
4	50	10	9.67×10^{8}
5	50	20	4.84×10^{9}
6	50	30	2.22×10^{9}
7	60	10	3.42×10^{9}
8	60	20	1.14×10^{10}
9	60	30	8.37×10^{9}

The quadratic equation obtained using coded values for the variables was given by:

$$R = 5.524 \times 10^{9} + 3.024 \times 10^{9}\,T + 1.026 \times 10^{9}\,t + 1.250 \times 10^{9}\,t \times T + 2.014 \times 10^{9}\,T^{2} - 4.269\,t^{2}$$

where R is the dependent response, i.e., the total sum of peaks areas of the standard mixture, T is the extraction temperature, t the extraction time, and T × t the interaction between extraction temperature and time.

All the coefficients were found to be statistically significant. A total of 95% of the total variance was explained by this model. To validate the model, three replicates in the central point were performed in order to estimate the experimental error and to detect any lack of fit. The response surface obtained by the use of DoE face-centred composite design is reported in Figure 1. The estimated optimum value range, obtained for the extraction

temperature using a response surface methodology, was at around 60 °C (Figure 1). In general, in the SPME experiments, the temperature parameter decreases the partition coefficient between the analyte and the extraction polymer [31]. However, on the other hand, it acts on the extraction by increasing the diffusion of the compounds and therefore increasing the extraction rate. The extraction time was found to have a statistically important positive effect on the total area and number of volatile compounds. The increase in extraction time improves the efficiency of extraction of compounds which have high boiling points and increases the detected peak area. However, the increase in extraction time does not show the same effect on compounds with low boiling point [32]. This could presumably explain the optimum values range found by response surface methodology for the extraction time, which was at intermediate time, i.e., around 20 min. Thus, further experiments were carried out to evaluate the flavour profile of the three wine samples by setting the extraction temperature and the extraction time of the SPME at 60 °C and 20 min, respectively.

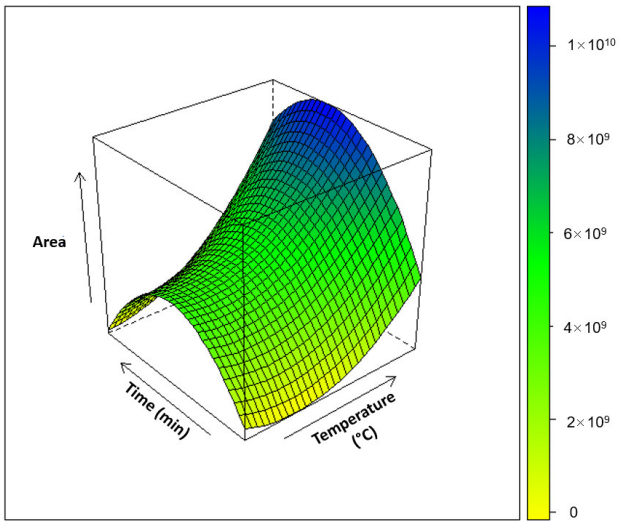

Figure 1. Response surface plot for the total sum of peaks areas of the standard mixture used as model for the HS-SPME optimization according to the DoE face-centred composite design (95% of the explained variance).

3.2. Wine Flavour Profiling

After the optimization of the HS-SPME/GC-MS method, the best conditions of the analysis were applied to the untargeted flavour profiling of the wines which had been obtained by fermentation with mixed cultures of yeasts and which were composed of the *S. cerevisiae* strain in association with the ND1 strain. This was tested both in free and immobilized cells, in comparison to the control, which was the experimental wine that had been obtained using single fermentation with the EC1118 strain. The SPME-GC/MS analysis allowed the identification of the 21 volatile organic compounds in the wine sample obtained by the mixed starters of *S. cerevisiae* and the free cells of *H. osmophila* (sample W1), which was a considerably greater number compared to the 8 compounds identified in the W2 wine sample that had been fermented with immobilized cells of the ND1 strain in co-culture with EC1118, and of the 6 compounds identified in the control (C), i.e., the wine sample obtained through the fermentation with *S. cerevisiae* alone. Such a difference in the number of VOCs detected in W1 and W2 wines could be due to a remarkable reduction in the enzymatic activity of yeasts, potentially caused by the diffusion limitations or the breakage of the microcapsules [33]. Figure 2 shows the total ion current chromatograms, obtained at optimized conditions, of W1 and W2 wine samples and of the control C.

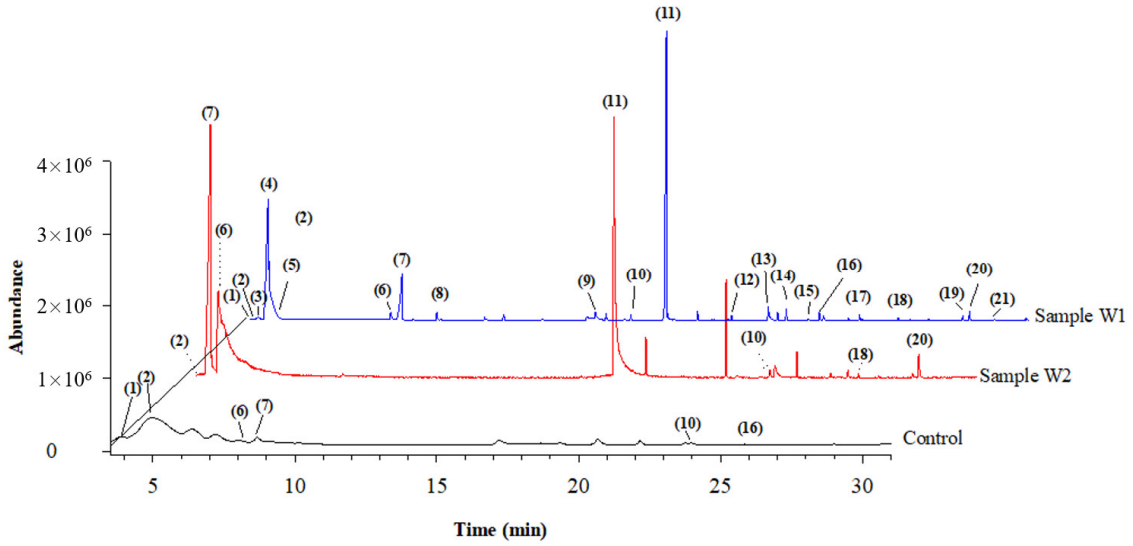

Figure 2. Total ion current chromatograms obtained by the HS-SPME/GC-MS analysis of the three wine samples, i.e., Aglianico wine obtained by fermentation with the mixed starters *S. cerevisiae* and free cells of *H. osmophila* (W1); Aglianico wine obtained by fermentation with the mixed starters *S. cerevisiae* and immobilized cells of *H. osmophila* (W2); Aglianico wine obtained by fermentation with *S. cerevisiae* alone (control C). SPME parameters: 100 µm PDMS fibre, extraction time 20 min, extraction temperature 60 °C, salinity 0.6 g of NaCl in 3 mL of sample volume.

All the volatile organic compounds detected in the three samples were identified by comparing their mass spectra with those available in the literature [34–36] and in the NIST electronic Mass Spectral Database. They belonged to five classes, namely higher alcohols, esters, volatile acids, terpenes, aldehydes and volatile phenols. The retention times, the quality match values with the NIST data and the main chemical-physical characteristics of the identified compounds are reported in Table 3, along with the occurrence or not of each VOC in the two wine samples and in the control. The wine obtained by co-inoculation of the free cells of *H. osmophila* and *S. cerevisiae* had a high number of higher alcohols, representing about the 44.9% of the total volatile organic compounds (Figure 3) and includ ethyl acetate (compound **2**), 2-butanol-3-methyl (compound **3**), 1-butanol, 3-methyl- (compound **4**), 1-heptanol (compound **5**), 2,3-butanediol (compound **8**), phenylethyl alcohol (compound **9**) and 1-dodecanol (compound **11**). Higher alcohols are also referred to as fusel alcohols and are alcohols which have more than two carbons in their chain; thus, they have higher molecular weights and higher boiling points than ethanol. The discussion regarding the role that these compounds play in improving the quality of oenological products remains controversial. Higher alcohols impart a range of organoleptic attributes, ranging from solvent-like to floral. which could positively influence wine flavour [37]. However, several authors attributed to higher alcohols the pungent and unpleasant character of some wines, especially when present at high levels [38,39]. This is the case of phenylethyl alcohol and 1-butanol, 3-methyl-, known also as isoamyl alcohol, both among the major fusel alcohols occurring in the W1 Aglianico wine analyzed in this study.

Table 3. Retention times, quality match values with the NIST library data, molecular formula, molecular weight, boiling points, odour threshold values (OTVs) of the organic volatile compounds identified in the Aglianico wine sample obtained by fermentation with the mixed starters *S. cerevisiae* and *H. osmophila* and in the control, i.e., Aglianico wine sample obtained by fermentation with *S. cerevisiae* alone.

Peak N.	Retention Time (min)	Compound Name	Quality Match	Molecular Formula	Molecular Weight (g/mol)	Boiling Point (°C)	OTV (mg/L)	Detected Compound *		
								W1	W2	C
(1)	1.52	Acetaldehyde	90	C_2H_4O	44.0262	20.20	0.21	+	+	+
(2)	2.41	Ethyl acetate	91	$C_4H_8O_2$	88.0524	77.11	3.90	+	+	+
(3)	3.93	2-butanol-3-methyl	89	$C_5H_{12}O$	88.1482	132.15	0.27	+	−	−
(4)	4.25	1-Butanol, 3-methyl-	90	$C_5H_{12}O$	88.1482	132.59	60	+	−	−
(5)	4.34	1-Heptanol	56	$C_7H_{16}O$	116.2013	178.79	0.21	+	−	−
(6)	8.63/2.31 **	1-Butanol, 3-methyl-, acetate	90	$C_7H_{14}O_2$	130.1849	144.39	0.16	+	+	+
(7)	8.89/4.0 ***	Acetic acid	91	$C_2H_4O_2$	60.0520	117.72	200	+	+	+
(8)	10.23	2,3-Butanediol	90	$C_4H_{10}O_2$	90.1210	201.45	0.10	+	–-	−
(9)	15.53	Phenylethyl Alcohol	91	$C_8H_{10}O$	122.1644	228.35	200	+	−	−
(10)	17.00	Octanoic acid, ethyl ester	91	$C_{10}H_{20}O_2$	172.2646	213.47	0.58	+	+	+
(11)	18.21	Acetic acid, 2-phenylethyl ester	90	$C_{10}H_{12}O_2$	164.2011	240.15	1.80	+	+	−
(12)	20.56	Decanoic acid, ethyl ester	94	$C_{12}H_{24}O_2$	200.3178	259.23	0.35	+	−	−
(13)	21.85	1-Dodecanol	94	$C_{12}H_{26}O$	186.3342	247.43	0.33	+	−	−
(14)	22.48	Phenol, 2,5 bis (1,1-dimethylethyl)	95	$C_{14}H_{22}O$	206.3239	352.59	−	+	−	−
(15)	23.26	Nerolidol	90	$C_{15}H_{26}O$	222.3663	363.36	1.00	+	−	−
(16)	23.65	Dodecanoic acid, ethyl ester	93	$C_{14}H_{28}O_2$	228.3709	304.99	6.30	+	−	+
(17)	25.07	Cyclododecane	92	$C_{12}H_{24}$	168.3190	250.85	−	+	−	−
(18)	26.42	Tetradecanoic acid, ethyl ester	98	$C_{16}H_{32}O_2$	256.4241	350.75	2.50	+	+	−
(19)	28.70	Ethyl 9-hexadecenoate	96	$C_{18}H_{34}O_2$	282.4614	400.67	0.03	+	−	−
(20)	28.93	Hexadecanoic acid, ethyl ester	99	$C_{18}H_{36}O_2$	284.4772	396.51	0.01	+	+	−
(21)	30.91	Linoleic acid ethyl ester	98	$C_{20}H_{36}O_2$	308.4986	450.59	−	+	−	−

*, + is for detected compound in the sample, − is for not detected compound in the sample. ** 1-Butanol, 3-methyl-, acetate eluted at 8.63 min in W1 and C sample, and at 2.31 min in W2 sample. *** Acetic acid eluted at 8.89 min in W1 and C sample, and at 4.0 min in W2 sample.

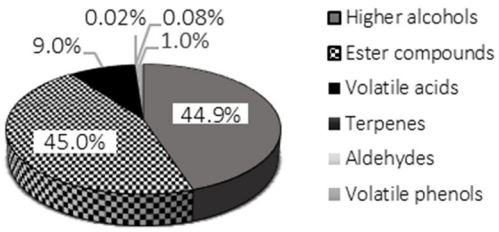

Figure 3. Pie chart reporting the percentage composition of higher alcohols, esters, volatile acids, terpenes, aldehydes and volatile phenols detected in the headspace of W1 wine sample, based on average chromatographic peaks areas.

In addition to the higher alcohols, a significant number of ester compounds were identified in the W1 wine sample, mainly ethyl esters. These specific compounds are biosynthesized through a condensation between ethanol and acyl-CoA, a process which is mediated by acyltransferases [40]. Ethyl esters and acetates represented the largest group (45%, Figure 3) of the total volatiles occurring in the wine obtained by mixed yeast inoculation. Many of these ester compounds are considered as varietal markers which allow the red wines to display varietal differentiation [41]. Moreover, they widely contribute to the fruity attributes of the oenological products. As evidence, the sensory evaluations reported in the literature revealed a stronger fruity character in wines fermented with mixed cultures than in control wines [42]. For example, 1-butanol, 3-methyl-, acetate (compound **6**), which was also identified in the wine sample that had been obtained by the co-inoculation of immobilized cells of *H. osmophila* and *S. cerevisiae* (W2) and in the control (C), was found to confer the typical banana flavour. This is characteristic of the *S. cerevisiae* metabolism; however, when present in excess, it could mask key varietal characters of wines [43]. Among the ester aromatic compounds detected in W1 sample, the most important was acetic acid, 2-phenylethyl ester (compound **11**). As described in previous studies, its concentration was higher in wines obtained with mixed cultures than in wines produced by *S. cerevisiae* pure cultures and it was modulated by changing the initial yeast ratio [42]. Acetic acid, 2-phenylethyl ester imparts "raspberry"- and 'honey'-like aromas to wine [44]. Ethyl esters of fatty acids also contribute with pleasant fruity and floral odours to wine aroma. Seven ethyl esters of straight-chain fatty acids with an even number of carbon atoms (C_8-C_{18}) were identified in sample W1, i.e., octanoic acid, ethyl ester (compound **10**), decanoic acid, ethyl ester (compound **12**), dodecanoic acid, ethyl ester (compound **16**), tetradecanoic acid, ethyl ester (compound **18**), ethyl 9-hexadecenoate (compound **19**), hexadecanoic acid, ethyl ester (compound **20**) and linoleic acid ethyl ester (compound **21**). The concentration of fatty acids ethyl esters is dependent on wine aging: it generally decreases as the storage time gets longer, especially at low pH and high temperature values [45,46]. Although their production has been shown to be lower when apiculate yeasts are inoculated, like those belonging to the genus *Hanseniaspora* [47], a greater presence of these compounds was found in this study in W1 wine compared to the control, in which only octanoic acid, ethyl ester and dodecanoic acid, ethyl ester occurred. As regards to W2 wine, octanoic acid, ethyl ester; acetic acid, 2-phenylethyl ester; tetradecanoic acid, ethyl ester; and hexadecanoic acid, ethyl ester were identified as representative compounds belonging to the ester class (Table 3). All the volatile fatty acids detected in the three samples occurred in their esterified form. The exception was acetic acid, which was also detected in its free form (compound **7**). It should be noted that a decrease in the retention time of acetic acid was observed for the W2 wine, which may have been due to the hydrolysis of ethyl acetate. As representative compounds belonging to the class of aldehydes and terpenes, acetaldehyde (compound **1**) and nerolidol (compound **15**) were, respectively, identified in the W1 wine. Moreover, the first one was also detected in the W2 wine sample

which had been fermented with immobilized cells of *H. osmophila* in co-cultures with *S. cerevisiae* and in the control. Acetaldehyde is derived from alcohol fermentation by yeasts. In this study, no intense chromatographic peaks were observed which related to other aldehydes in the total ion current chromatograms of the three analyzed samples. This was most likely because they were involved in several reactions with wine phenolics, whose products impacted wine colour, flavour and astringency [48]. Nerolidol (compound **15**), a compound with a characteristic floral odour similar to that of rose [49], was not detected in the W2 sample, nor was it found in the control. This occurred because, in general, the presence of flavour compounds belonging to the terpenes class is due to some specific enzymatic activities of the yeasts, usually those activities linked to non-*Saccharomyces* species which can contribute to increasing the sensory profile of the wine [50]. Instead, compound **14**, i.e., phenol, 2,5 bis (1,1-dimethylethyl) was the only volatile phenol detected in the co-fermented wine W1. Such a compound has been previously identified by Lu et al. [51] in the Changyu wine. It is produced by wild yeast and, at concentrations higher than 600 μg/L, it confers a fishy smell to wine [52]. As regards saturated cyclic alkanes, a cyclododecane (compound **17**), previously found in Nero di Troia wine by Baiano et al. [53], has been detected in W1 wine sample, probably as a contaminant.

4. Conclusions

If properly optimized through experimental design, headspace solid-phase microextraction, coupled to GC-MS analysis, ensures a comprehensive characterization of the aroma compounds responsible for wine complexity. Here, a face-centred composite experimental design matrix and response surface methodology were applied to designing the experiments and evaluating the interactive effects of the two studied parameters, i.e., extraction time and extraction temperature. The optimum conditions which were suggested by the second-order polynomial regression model for the obtention of higher amounts of VOCs were 20 min and 60 °C. The optimized HS-SPME/GC-MS-based method allowed us to ascertain the flavour complexity of wine which had been obtained by controlled multi-starter fermentations between non-*Saccharomyces* and *Saccharomyces* strains. *H. osmophila*, used as free cells in co-inoculated fermentation with a commercial strain of *S. cerevisiae*, proved able to positively modulate the flavour profile of Aglianico wine as the number of volatile organic compounds detected in the headspace of the wine sample was greatly higher (21 compounds) compared to the wine control, which had been obtained by fermentation with *S. cerevisiae* alone (6 compounds).

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References

1. Stanco, M.; Lerro, M.; Marotta, G. Consumers' preferences for wine attributes: A best-worst scaling analysis. *Sustainability* **2020**, *12*, 2819. https://doi.org/10.3390/su12072819.
2. Fischer, U. Wine Aroma. In *Flavours and Fragrances*; Springer: Berlin/Heidelberg, Germany, 2007; pp. 241–267.
3. Ciani, M.; Comitini, F. Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* **2015**, *1*, 1–6. https://doi.org/10.1016/j.cofs.2014.07.001.

4. Kosseva, M.R. Immobilization of Microbial Cells in Food Fermentation Processes. *Food Bioprocess Technol.* **2011**, *4*, 1089–1118. https://doi.org/10.1007/s11947-010-0435-0.
5. Acquavia, M.A.; Pascale, R.; Pappalardo, I.; Santarsiero, A.; Martelli, G.; Bianco, G. Characterization of quercetin derivatives in crossing combination of habanero white and capsicum annuum peppers and of anti-inflammatory and cytotoxic activity. *Separations* **2021**, *8*,90. https://doi.org/10.3390/separations8070090.
6. Pascale, R.; Acquavia, M.A.; Onzo, A.; Cataldi, T.R.I.; Calvano, C.D.; Bianco, G. Analysis of surfactants by mass spectrometry: Coming to grips with their diversity. *Mass Spectrom. Rev.* **2021**, 1–32. https://doi.org/10.1002/mas.21735.
7. Acquavia, M.A.; Pascale, R.; Foti, L.; Carlucci, G.; Scrano, L.; Martelli, G.; Brienza, M.; Coviello, D.; Bianco, G.; Lelario, F. Analytical methods for extraction and identification of primary and secondary metabolites of apple (*Malus domestica*) fruits: A review. *Separations* **2021**, *8*, 91. https://doi.org/10.3390/separations8070091.
8. Onzo, A.; Acquavia, M.A.; Cataldi, T.R.I.; Ligonzo, M.; Coviello, D.; Pascale, R.; Martelli, G.; Bondoni, M.; Scrano, L.; Bianco, G. Coceth sulfate characterization by electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2020**, *34*, e8884. https://doi.org/10.1002/rcm.8884.
9. Abbott, N.; Etiévant, P.; Langlois, D.; Lesschaeve, I.; Issanchou, S. Evaluation of the Representativeness of the Odor of Beer Extracts Prior to Analysis by GC Eluate Sniffing. *J. Agric. Food Chem.* **1993**, *41*, 777–780. https://doi.org/10.1021/jf00029a019.
10. Ortega-Heras, M.; González-SanJosé, M.L.; Beltrán, S. Aroma composition of wine studied by different extraction methods. *Anal. Chim. Acta* **2002**, *458*, 85–93. https://doi.org/10.1016/S0003-2670(01)01526-4.
11. Castro, R.; Natera, R.; Durán, E.; García-Barroso, C. Application of solid phase extraction techniques to analyse volatile compounds in wines and other enological products. *Eur. Food Res. Technol.* **2008**, *228*, 1–18. https://doi.org/10.1007/s00217-008-0900-4.
12. López, R.; Aznar, M.; Cacho, J.; Ferreira, V. Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. *J. Chromatogr. A* **2002**, *966*, 167–177. https://doi.org/10.1016/S0021-9673(02)00696-9.
13. De-La-fuente-blanco, A.; Ferreira, V. Gas chromatography olfactometry (Gc-o) for the (semi)quantitative screening of wine aroma. *Foods* **2020**, *9*, 1892. https://doi.org/10.3390/foods9121892.
14. Sagratini, G.; Maggi, F.; Caprioli, G.; Cristalli, G.; Ricciutelli, M.; Torregiani, E.; Vittori, S. Comparative study of aroma profile and phenolic content of Montepulciano monovarietal red wines from the Marches and Abruzzo regions of Italy using HS-SPME-GC-MS and HPLC-MS. *Food Chem.* **2012**, *132*, 1592–1599. https://doi.org/10.1016/j.foodchem.2011.11.108.
15. Perestrelo, R.; Silva, C.L.; Silva, P.; Câmara, J.S. Establishment of the volatile signature of wine-based aromatic vinegars subjected to maceration. *Molecules* **2018**, *23*, 499. https://doi.org/10.3390/molecules23020499.
16. Tao, Y.S.; Li, H.; Wang, H.; Zhang, L. Volatile compounds of young Cabernet Sauvignon red wine from Changli County (China). *J. Food Compos. Anal.* **2008**, *21*, 689–694. https://doi.org/10.1016/j.jfca.2008.05.007.
17. Bianco, G.; Novario, G.; Zianni, R.; Cataldi, T.R.I. Comparison of two SPME fibers for the extraction of some off-flavor cork-taint compounds in bottled wines investigated by GC-HRMS. *Anal. Bioanal. Chem.* **2009**, *393*, 2019–2027. https://doi.org/10.1007/s00216-009-2632-0.
18. Fiorini, D.; Pacetti, D.; Gabbianelli, R.; Gabrielli, S.; Ballini, R. A salting out system for improving the efficiency of the headspace solid-phase microextraction of short and medium chain free fatty acids. *J. Chromatogr. A* **2015**, *1409*, 282–287. https://doi.org/10.1016/j.chroma.2015.07.051.
19. Parameswaran, R.; Box, G.E.P.; Hunter, W.G.; Hunter, J.S. Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building. *J. Mark. Res.* **1979**, *16*, 291. https://doi.org/10.2307/3150696.
20. Bouaid, A.; Ramos, L.; Gonzalez, M.J.; Fernández, P.; Cámara, C. Solid-phase microextraction method for the determination of atrazine and four organophosphorus pesticides in soil samples by gas chromatography. *J. Chromatogr. A* **2001**, *939*, 13–21. https://doi.org/10.1016/S0021-9673(01)01332-2.
21. Varrone, C.; Giussani, B.; Izzo, G.; Massini, G.; Marone, A.; Signorini, A.; Wang, A. Statistical optimization of biohydrogen and ethanol production from crude glycerol by microbial mixed culture. *Int. J. Hydrogen Energy* **2012**, *37*, 16479–16488. https://doi.org/10.1016/j.ijhydene.2012.02.106.
22. Leardi, R. Experimental design in chemistry: A tutorial. *Anal. Chim. Acta* **2009**, *652*, 161–172. https://doi.org/10.1016/j.aca.2009.06.015.
23. Boscaino, F.; Ionata, E.; La Cara, F.; Guerriero, S.; Marcolongo, L.; Sorrentino, A. Impact of *Saccharomyces cerevisiae* and *Metschnikowia fructicola* autochthonous mixed starter on Aglianico wine volatile compounds. *J. Food Sci. Technol.* **2019**, *56*, 4982–4991. https://doi.org/10.1007/s13197-019-03970-9.
24. Alba, V.; Anaclerio, A.; Gasparro, M.; Caputo, A.R.; Montemurro, C.; Blanco, A.; Antonacci, D. Ampelographic and molecular characterisation of Aglianico accessions (*Vitis vinifera* L.) collected in Southern Italy. *S. Afr. J. Enol. Vitic.* **2011**, *32*, 164–173. https://doi.org/10.21548/32-2-1376.
25. Mok, C.; Song, K.T.; Park, Y.S.; Lim, S.; Ruan, R.; Chen, P. High hydrostatic pressure pasteurization of red wine. *J. Food Sci.* **2006**, *71*, M265–M269. https://doi.org/10.1111/j.1750-3841.2006.00145.x.
26. Alberico, G.; Capece, A.; Mauriello, G.; Pietrafesa, R.; Siesto, G.; Garde-cerd, T.; Maresca, D.; Romano, R.; Romano, P. Influence of Microencapsulation on Fermentative Behavior of Hanseniaspora osmophila in Wine Mixed Starter Fermentation. *Fermentation* **2021**, *7*, 112.

27. Leardi, R.; Melzi, C.; Polotti, G. CAT (Chemometric Agile Tool). http://gruppochemiometria.it/index.php/software. Accessed on June 30th 2021.
28. Chidi, B.S.; Bauer, F.F.; Rossouw, D. Organic acid metabolism and the impact of fermentation practices on wine acidity–A review. *S. Afr. J. Enol. Vitic.* **2018**, *39*, 315–329. https://doi.org/10.21548/39-2-3172.
29. Belda, I.; Ruiz, J.; Esteban-Fernández, A.; Navascués, E.; Marquina, D.; Santos, A.; Moreno-Arribas, M.V. Microbial contribution to Wine aroma and its intended use for Wine quality improvement. *Molecules* **2017**, *22*, 189. https://doi.org/10.3390/molecules22020189.
30. Romano, P.; Caruso, M.; Capece, A.; Lipani, G.; Paraggio, M.; Fiore, C. Metabolic diversity of Saccharomyces cerevisiae strains from spontaneously fermented grape musts. *World J. Microbiol. Biotechnol.* **2003**, *19*, 311–315. https://doi.org/10.1023/A:1023666510737.
31. Rocha, S.; Ramalheira, V.; Barros, A.; Delgadillo, I.; Coimbra, M.A. Headspace solid phase microextraction (SPME) analysis of flavor compounds in wines. Effect of the matrix volatile composition in the relative response factors in a wine model. *J. Agric. Food Chem.* **2001**, *49*, 5142–5151. https://doi.org/10.1021/jf010566m.
32. Ho, C.W.; Wan Aida, W.M.; Maskat, M.Y.; Osman, H. Optimization of headspace solid phase microextraction (HS-SPME) for gas chromatography mass spectrometry (GC-MS) analysis of aroma compound in palm sugar (*Arenga pinnata*). *J. Food Compos. Anal.* **2006**, *19*, 822–830. https://doi.org/10.1016/j.jfca.2006.05.003.
33. Benucci, I.; Cecchi, T.; Lombardelli, C.; Maresca, D.; Mauriello, G.; Esti, M. Novel microencapsulated yeast for the primary fermentation of green beer: Kinetic behavior, volatiles and sensory profile. *Food Chem.* **2021**, *340*, 127900. https://doi.org/10.1016/j.foodchem.2020.127900.
34. De Hoffmann, E.; Stroobant, V. *Mass Spectrometry Principles and Applications*; John & Wiley and Sons: Hoboken, NJ, USA, 2007.
35. Fu, M.; Duan, P.; Gao, J.; Kenttämaa, H.I. Ion-molecule reactions for the differentiation of primary, secondary and tertiary hydroxyl functionalities in protonated analytes in a tandem mass spectrometer. *Analyst* **2012**, *137*, 5720–5722. https://doi.org/10.1039/c2an35986c.
36. Alves, S.P.; Tyburczy, C.; Lawrence, P.; Bessa, R.J.B.; Thomas Brenna, J. Acetonitrile covalent adduct chemical ionization tandem mass spectrometry of non-methylene-interrupted pentaene fatty acid methyl esters. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 1933–1941. https://doi.org/10.1002/rcm.5065.
37. De-La-Fuente-Blanco, A.; Sáenz-Navajas, M.P.; Ferreira, V. On the effects of higher alcohols on red wine aroma. *Food Chem.* **2016**, *210*, 107–114. https://doi.org/10.1016/j.foodchem.2016.04.021.
38. San-juan, F.; Ferreira, V.; Cacho, J.; Escudero, A.; Prolabo, B.D.H. Quality and Aromatic Sensory Descriptors (Mainly Fresh and Dry Fruit Character) of Spanish Red Wines can be Predicted from their Aroma-Active Chemical Composition. *J. Agric. Food Chem.* **2011**, *59*, 7916–7924.
39. Saenz-Navajas, M.-P.; Avizcuri, J.-M.; Ballester, J.; Fernandez-Zurbano, P.; Ferreira, V.; Dominique Peyron, D.V. Sensory-active compounds influencing wine experts' and consumers' perception of red wine intrinsic quality. *Food Sci. Technol.* **2015**, *60*, 400–411. https://doi.org/10.1016/j.lwt.2014.09.026.
40. Costello, P.J.; Siebert, T.E.; Solomon, M.R.; Bartowsky, E.J. Synthesis of fruity ethyl esters by acyl coenzyme A: Alcohol acyltransferase and reverse esterase activities in Oenococcus oeni and Lactobacillus plantarum. *J. Appl. Microbiol.* **2013**, *114*, 797–806. https://doi.org/10.1111/jam.12098.
41. Antalick, G.; Šuklje, K.; Blackman, J.W.; Meeks, C.; Deloire, A.; Schmidtke, L.M. Influence of Grape Composition on Red Wine Ester Profile: Comparison between Cabernet Sauvignon and Shiraz Cultivars from Australian Warm Climate. *J. Agric. Food Chem.* **2015**, *63*, 4664–4672. https://doi.org/10.1021/acs.jafc.5b00966.
42. Viana, F.; Gil, J.V.; Vallés, S.; Manzanares, P. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of Hanseniaspora osmophila and Saccharomyces cerevisiae. *Int. J. Food Microbiol.* **2009**, *135*, 68–74. https://doi.org/10.1016/j.ijfoodmicro.2009.07.025.
43. Plata, C.; Millán, C.; Mauricio, J.C.; Ortega, J.M. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiol.* **2003**, *20*, 217–224. https://doi.org/10.1016/S0740-0020(02)00101-6.
44. Swiegers, J.H.; Bartowsky, E.J.; Henschke, P.A.; Pretorius, I.S. Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* **2005**, *11*, 139–173. https://doi.org/10.1111/j.1755-0238.2005.tb00285.x.
45. Díaz-Maroto, M.C.; Schneider, R.; Baumes, R. Formation pathways of ethyl esters of branched short-chain fatty acids during wine aging. *J. Agric. Food Chem.* **2005**, *53*, 3503–3509. https://doi.org/10.1021/jf048157o.
46. Câmara, J.S.; Alves, M.A.; Marques, J.C. Changes in volatile composition of Madeira wines during their oxidative ageing. *Anal. Chim. Acta* **2006**, *563*, 188–197. https://doi.org/10.1016/j.aca.2005.10.031.
47. Herraiz, T.; Reglero, G.; Herraiz, M.; Martin-Alvarez, P.J.; Cabezudo, M.D. The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. *Am. J. Enol. Vitic.* **1990**, *41*, 313–318. https://doi.org/10.1111/j.1365-2621.1996.tb10971.x.
48. Han, G.; Webb, M.R.; Waterhouse, A.L. Acetaldehyde reactions during wine bottle storage. *Food Chem.* **2019**, *290*, 208–215. https://doi.org/10.1016/j.foodchem.2019.03.137.
49. Drtilová, T.; Ďurčanská, K.; Machyňáková, A.; Špánik, I.; Klempová, T.; Furdíková, K. Impact of different pure cultures of *Saccharomyces cerevisiae* on the volatile profile of Cabernet Sauvignon rosé wines. *Czech J. Food Sci.* **2020**, *38*, 94–102. https://doi.org/10.17221/338/2018-CJFS.
50. Romano, P.; Braschi, G.; Siesto, G.; Patrignani, F. Role of Yeasts on the Sensory Component of Wines. *Foods* **2022**, *11*, 1921.

51. Lu, L.; Mi, J.; Chen, X.; Luo, Q.; Li, X.; He, J.; Zhao, R.; Jin, B.; Yan, Y.; Cao, Y. Analysis on volatile components of co-fermented fruit wines by *Lycium ruthenicum* murray and wine grapes. *Food Sci. Technol.* **2021**, *42, 1-8*. https://doi.org/10.1590/fst.12321.
52. Zhang, X.K.; Lan, Y.B.; Zhu, B.Q.; Xiang, X.F.; Duan, C.Q.; Shi, Y. Changes in monosaccharides, organic acids and amino acids during Cabernet Sauvignon wine ageing based on a simultaneous analysis using gas chromatography–mass spectrometry. *J. Sci. Food Agric.* **2018**, *98*, 104–112. https://doi.org/10.1002/jsfa.8444.
53. Baiano, A.; Mentana, A.; Quinto, M.; Centonze, D.; Previtali, M.A.; Varva, G.; Del Nobile, M.A.; De Palma, L. Volatile composition and sensory profile of wines obtained from partially defoliated vines: The case of Nero di Troia wine. *Eur. Food Res. Technol.* **2017**, *243*, 247–261. https://doi.org/10.1007/s00217-016-2740-y.