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Use of potassium polyaspartate on white wines: Interaction with proteins and aroma compounds

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ABSTRACT

The precipitation of tartaric salts represents one of the main visual sensory faults of white wines. It can be prevented by cold stabilization or adding some adjuvants, such as potassium polyaspartate (KPA). KPA is a biopolymer that can limit the precipitation of tartaric salts linking the potassium cation, however, it could interact also with other compounds affecting wine quality. The present work aims to study the effect of potassium polyaspartate on proteins and aroma compounds of two white wines, at different storage temperatures (4 °C and 16 °C). The KPA addition showed positive effects on the quality of wines, with a significant decrease of unstable proteins (up to 92%), also related to better wine protein stability indices. A Logistic function well described the effect of KPA and storage temperature on protein concentration ($R^2 > 0.93$; NRMSD: 1.54–3.82%). Moreover, the KPA addition allowed the preservation of the aroma concentration and no adversely effects were pointed out. Alternatively to common enological adjuvants, KPA could be considered a multifunctional product against tartaric and protein instability of white wines, avoiding adverse effects on their aroma profile.

1. Introduction

White wines can be affected by several sensory faults, inducing a decrease of wine quality, consumer reject, and economic losses for the wineries. Those sensory defects occur due to several factors and processing conditions that favors their formation (Cosme et al., 2021).

Generally, the most unmanageable defects occur after wine bottling. During storage, commercialization, and selling stages, several external factors can affect wine stability, such as temperature and light exposures (Echave et al., 2021). Temperature is considered one of the key factors affecting two undesired visual sensory faults of white wines: the precipitation of bitartrate salts, and the formation of protein haze (Cosme et al., 2020, 2021).

The precipitation of tartaric salts in form of crystals at the bottom of the bottles can be prevented by several techniques. The "subtractive" techniques involve the reduction of the concentration of ions responsible for the tartaric precipitation in wines; instead, the "additive" approaches use protective colloids or crystallization inhibitors that can be added to the wine (Martínez-Pérez et al., 2020).

The cold stabilization is the most widespread method used by wineries. This practice involves decreasing the wine's temperature near its freezing point and storing it in an isothermal tank for a variable time of 1–3 weeks, to promote the precipitation of tartaric salts before bottling. Despite its effectiveness, this technique shows several drawbacks: co-precipitation and losses of wine color and aroma compounds, long treatment times, high economic costs, and environmental problems related to waste generation (Filipe-Ribeiro et al., 2021; Lasanta & Gómez, 2012; Martínez-Pérez et al., 2020).

Electrodialysis (ED) and ion exchangers (IE) are alternative subtractive techniques. ED is a separation/concentration process of ions, through selective membranes and the application of an electric field between two electrodes. Instead, IE use specific insoluble gel matrices able to replace the potassium ions with hydrogen or sodium ions and, in this way, lowering the concentration of one of the substrates of the reaction (Ibeas et al., 2015). Both of them have some disadvantages: high initial investment, and operating costs, and they are sometimes excessive selective. Moreover, the use of ion exchangers is not recommended when the instability is due to an excessive tartaric acid content (Bosso et al., 2015).

The additive processes, instead, involve the addition of several adjuvants: metatartaric acid, carboxymethylcellulose (CMC), mannoproteins, gum Arabic or potassium polyaspartate (KPA).

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Potassium polyaspartate was approved by OIV in 2016 and its use was subsequently regulated also by Europe Union (Commission Delegated Regulation (EU) 2017/1961, 2017). KPA is a biopolymer obtained from condensation reaction of L-Aspartic acid, that at wine pH presents a negative charge, allowing to link the potassium cation and limiting the precipitation of tartaric salts. Several investigations revealed no toxicity effects on human health (Galbusera et al., 2017) and a stabilization capacity similar to CMC, but more persistent over time.

The enological adjuvants severely affect the wine resilience and the sensory profile. For example, the bentonite is used for the interaction with haze-forming proteins but it could interact with other chemical compounds, such as phenols and aroma compounds (Lambri et al., 2016).

Previous research already reported that KPA do not interacts with polyphenols, tannins or anthocyanins, preserving the color or pigmentations of the wine (Bosso, Motta, Panero, Lucini, et al., 2020; Bosso, Motta, Panero, Petrozziello, et al., 2020). Further investigations are needed to better understand the effect of KPA addition on other chemical compounds affecting the sensorial characteristics of white wines.

In view of the recent approval by European Commission of KPA as enological adjuvant for the tartaric stabilization of white wines, and its chemical properties, the aim of the present work was to study the effect of potassium polyaspartate on proteins and aroma compounds of two different white wines. Studies were preformed considering the maximum concentration allowed by the European legislation (100 mg/L) and a comparison between different storage temperatures and times. The effect on protein fractions was evaluated by several analytical determinations, such as turbidity, protein charge neutralization test, heat test, cold tannin test, surface electrical charge, and HPLC quantitative analysis. Finally, the effect on aroma profile of wines was determined by GC–MS analysis to highlight some possible interactions involved by KPA addition.

2. Material and methods

2.1. Reagents and solvents

Ethanol, methanol, and acetonitrile were of analytical grade (purity $>$ 99%) and purchased from Sigma Aldrich Co. (Milan, Italy). The chemicals used, which include polydiallydimetyl ammonium chloride solution (Poly- DADMAC 20 wt%) and trifluoroacetic acid were of analytical grade and purchased from Sigma Aldrich Co. (Milan, Italy). The potassium polyaspartate for enological uses was purchased from Ever S.r.L (Pramaggiore, Italy).

2.2. Wine sample

Two white wines, Cortese and Lugana varieties (Vitis vinifera L.), produced in the 2020 vintage, were chosen from a winery in the Piemonte and the Veneto region (Italy), respectively. The Cortese wine had the initial physicochemical characteristics of alcohol 12.5%, pH = 3.49, total acidity 5.3 g/L, reducing sugars 3.8 g/L, and total SO_2 60 mg/L. Instead, the Lugana wine had the following characteristics: alcohol 11.2%, pH = 3.32, total acidity 6.8 g/L, reducing sugars 4.3 g/L, and total SO_2 45 mg/L.

2.3. Sample treatments

An aliquot (20 L) of Cortese (CO) and Lugana (LU) wine was treated with the maximum concentration of potassium polyaspartate (100 mg/L). A further aliquot (20 L) of both wines was considered as control, in which no KPA addition was added.

The untreated (C) and treated (KPA) samples were bottled in 750 mL bottles, stored at two different temperatures (T-amb = 16 °C and T4 = 4 °C) and they were monitored at different storage times (10, 30, 60 and 90 days). All the experiments were carried out in triplicate.

2.4. Analytical methods

2.4.1. Turbidity determination

All samples were filtered in a 0.45 μm syringe filter before turbidity measurements, carried out in a AL250T-IR turbidimeter (Acqualytic, Dortmund, Germany). The results are expressed as nephelometric turbidity unit (NTU).

2.4.2. Protein charge neutralization test (PCN)

The protein charge neutralization (PCN) test is a commercial rapid specific method (Protocheck ®) for the evaluation of protein instability (Celotti & Martellozzo, 2006). Each sample was filtered in a 0.45 μm syringe filter and added to the tubes containing a liquid solution of anionic compounds, which reacted with the wine proteins. The turbidity was measured initially and after 60 s of mixture agitation, and repeated 5 times. The results were then calculated with the following equation:

$$PCN = NTU2 - (NTU1 / 1.5) \quad (1)$$

where *PC* is Protein Charge Neutralization (PCN) value, *NTU1* is the initial turbidity value, and *NTU2* is the value of turbidity after PCN test.

2.4.3. Heat stability test (HT)

Ten milliliters of sample were filtered through 0.45 μm filters and sealed in test tubes with screw caps. The tubes were heated at 80 ∘C for 30 min (Gabrielli et al., 2016; McRae et al., 2018). Afterward, the sample was left to cool at room temperature. The formula used to obtain this result is the difference between the turbidity after heat exposure and the initial turbidity, considering the data with a difference of $\geq$ 5.5 as unstable wine (Moreno-Arribas & Polo, 2009):

$$HT = NTUH - NTU1 \quad (2)$$

where *HT* is the heat test, *NTUH* is the value of turbidity after heat treatment, and *NTU1* is the initial turbidity value.

2.4.4. Cold tannin test (CTT)

The samples were filtered in a 0.45 μm syringe filter; subsequently, 100 μL of chestnut tannin–ethanol solution (5% w/v) was added. Turbidity was measured using a AL250T-IR turbidimeter (Acqualytic, Dortmund, Germany) before and after the tannin solution addition. The results of cold tannin test were measured by the following equation:

$$CTT = NTUT - NTU1 \quad (3)$$

where *CTT* is the cold tannin test value, *NTU1* is the initial value of turbidity, and *NTUT* is the value of turbidity after tannin addition.

2.4.5. Surface electric charge (SEC)

The surface electrical charge (SEC) was determined with a particle size detector (Mütek PCD 03, Mutek Analytical GmbH, Herrsching, Germany). The wine samples showed an initial negative charge at pH 4; therefore, a titration organic cationic polydiallydimetyl ammonium chloride (PolyDADMAC) solution (10–3 N) was used to quantify the surface electrical charge. PolyDADMAC solution was continuously added to 10 mL of wine samples until the charge equilibrium was achieved. Surface electrical charge is expressed as milliequivalents per liter (meq/L) of negative charges present in the sample, and calculated by the equation:

$$SEC = \frac{\left(mL_{PolyDADMAC}\right)}{1000} \times 100$$

2.4.6. Protein determination by high performance liquid chromatography (HPLC)

Wine proteins were precipitated from 4 mL of wine sample, adding 20 mL of ethanol (96% v/v). Subsequently, 10 mL of the obtained solution was subjected to centrifugation at 3000 rpm, the ethanol was

completely removed, and the proteins were dissolved in 1 mL of milli-Q water. HPLC analysis was performed on an LC-2010 AHT liquid chromatographic system (Shimadzu, Kyoto, Japan), equipped with an integrated autosampler and UV–Vis detector. Compound separation was achieved with a 4.6 × 250 mm Vydac C8 column (Altech, Milan, Italy), coupled with a 4.6 × 5 mm precolumn (Altech, Milan, Italy) with the same stationary phase, and thermostated at 35 °C. The mobile phase was composed of 83% (v/v) solvent A (0.1% trifluoroacetic acid in 8% acetonitrile solution) and 17% (v/v) solvent B (0.1% trifluoroacetic acid in 80% acetonitrile solution). A linear gradient was set as follows: solvent B was increased from 17% to 49% in the first 7 min, from 49% to 57%from7to15min, from57%to65%from15to16min, from65%to 81% from 16 to 30 min, and then held at 81% for 5 min before re- equilibrating the column in the starting conditions for an additional 6 min. The injection volume was 100 μL and the flow rate was set to 1 mL/ min.

The peaks were detected at 210 nm and qualitative analysis was carried out as reported in literature (Marangon et al., 2009): peaks with a retention time between 9 and 12 min were assigned to the TL protein classes, whereas peaks eluted from 18.5 and 24.5 min were assumed to be chitinases. Protein quantification was done through a calibration curve of Bovine Serum Albumin (BSA) at different concentration (50–1000 ppm).

2.4.7. SPME analysis GC–MS aroma compounds

The volatile composition of control and treated samples was characterized by SPME–GC–MS. Analyses were carried out using a GC- 17A gas chromatograph equipped with a QP-5000 mass spectrometer (Shimadzu, Kyoto, Japan). Wine samples (10 mL) were introduced in 50 mL amber glass vials sealed with PTFE/silicone septa, with 3 g of NaCl and 100 L of an ethyl-heptanoate standard solution (0.09384 g/L).

Vials were pre-conditioned for 15 min at 40 °C before microextraction, and SPME was run at the same temperature for 15 min, using a 2 cm 50/30 lm divinylbenzene/carboxen/polydimethyl- siloxane fiber (Supelco, Bellefonte, PA, USA). A J&W DB-Wax capillary column, 30 m × 0.25 mm i.d., 0.5 μm film thickness (Agilent Technologies Inc., Santa Clara, CA, USA) was used for the GC separation, with the following operating conditions: 40 °C for 5 min, then 4 °C min^{-1}, up to 240 °C, with a final holding of time of 10 min. Injection was performed in splitless mode with 60 s of splitless time; injection port and transfer line were set at 250 and 280 °C respectively. Carrier gas was helium, at a linear flow rate of 0.9 mL/min.

The mass spectrophotometer was set in SCAN mode at the range *m/z* ratio 30–350. The peak identification was carried out through the software library (Agilent MassHunter Qualitative Analysis B.07.00) and the comparison of literature data (https://webbook.nist.gov/chemistry/). The area of each aroma compound peak was then related to the internal standard one.

2.5. Mathematical modelling

The protein content of untreated (C) and treated samples (KPA) during storage at different temperatures was mathematically described by a Logistic function. The model equation was:

$$Y = \frac{K}{(1 - a \cdot e^{-b \cdot t})}$$

Where Y is the protein content (mg/L), t is the storage time (days), K, a and b are the estimated model coefficients. The values of the model parameters and graph plots were calculated using Matlab 2019b (MathWorks, Inc., USA). The agreement between the experimental values was assessed by means of correlation co-efficients (R^2 and R^2_{adj}) and the normalized root means squared deviation (NRMSD) criterion, which is defined as:

$$NRMSD = \frac{RMSD}{exp_{max}} = \frac{\sqrt{(1/n) \cdot \sum_{p=1}^{n} (exp_p - mod_p)^2}}{exp_{max}}$$

where n is the number of experimental points composing a graph curve, exp_p is the experimental value at point p, mod_p is the model value at point p, and exp mac is the maximum within the n experimental values.

2.6. Statistical analysis

All experiments and analysis were performed in triplicate and results are expressed as mean ± standard deviation. Minitab 17 software (Minitab Inc., State College, Pennsylvania, US) was used for statistical analysis by one-way analysis of variance (ANOVA, with Tukey's honest significant difference (HSD) multiple comparison test) with the level of significance set at $p < 0.05$. The principal component analysis (PCA) and a regression analysis were adopted for the main aroma compounds categories identified on Cortese and Lugana wines. The regression equation for total sum of relative area of aroma compounds was:

$$Y = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_3 + b_{12} \cdot X_1 \cdot X_2 + b_{13} \cdot X_1 \cdot X_3 + b_{23} \cdot X_2 \cdot X_3 + b_{123} \cdot X_1 \cdot X_2 \cdot X_3$$

where Y represents the response variable, b_0 is a constant, b_i, b_{ii} and b_{ij} are the linear, quadratic and interactive coefficients, respectively. The adequacy of the polynomial model was expressed by the coefficient of multiple determinations (R^2 and R^2-adj), and analysis of variance (ANOVA) was employed to determine the significance of the model. Statistical significance of the model and the model variables was determined at $p < 0.05$.

3. Results and discussion

3.1. Effect on turbidity

The turbidity of untreated (C) and treated samples (KPA), at ambient and refrigerated (T4) temperature, was measured during 90 days of storage. The results are reported in Table 1. The wine turbidity is affected by an heterogeneous group of insoluble particles, wine colloids, and partially soluble macromolecules (Ribéreau-Gayon et al., 2006). Wine colloids are charged particles, constituted by condensed phenols, colloidal colouring matter, proteins, and carbohydrates, assembled by cohesive intermolecular forces (Osorio-Macías et al., 2020).

The Cortese wine showed higher initial turbidity (18.43 ± 1.86 NTU), than Lugana (8.64 ± 0.22 NTU). The turbidity significantly decreased after 10 days of storage, independently of KPA addition and temperature. The storage temperature affects the clarifications phenomena of wine, and lower temperatures increase the wine macromolecules sedimentation rate (Vernhet, 2022). As reported in Table 1, the Cortese wine showed the lowest turbidity for untreated (0.47 ± 0.20 NTU) and treated samples (0.48 ± 0.16 NTU), after 90 days of storage at 4 °C (T4). Moreover, the KPA addition on Cortese wine induced a higher turbidity at 10 days, compared to the control samples (C and C-T4). The higher turbidity of wines after KPA addition has been already reported. The turbidity increase was probably due to the interaction between KPA and certain molecules, such as proteins and other colloidal compounds (Bosso, Motta, Panero, Lucini, et al., 2020; Bosso, Motta, Panero, Petrozziello, et al., 2020), that could affect also sedimentation rate.

The experimental trials on Lugana wine didn't highlight the same effects of KPA addition and storage temperature. The storage at 4 °C decreased the turbidity only for control samples at any analysis step and the KPA addition didn't induce an increase of turbidity, as observed for Cortese wine. Not only, but turbidity was always lower in samples supplemented with KPA at room temperature and indistinguishable at 4 °C. The wine is a complex mixture of heterogenous compounds, and different molecular interactions and phenomena could be observed after KPA addition. For instance, Lugana wine showed low turbidity (1 NTU)

Table 1
Turbidity of untreated (C) and treated (KPA) samples of Cortese and Lugana wine, at different storage temperature (16 °C and 4 °C), and time (10, 30, 60, and 90 days).

	TURBIDITY (NTU)			
CORTESE wine	**C**	**C - T4**	**KPA**	**KPA - T4**
t = 0 days	18.43 ± 1.86 a A*	18.43 ± 1.86 a A	18.43 ± 1.86 a A	18.43 ± 1.86 a A
t = 10 days	2.80 ± 0.78b BC	1.83 ± 0.97b C	6.15 ± 1.92b AB	8.34 ± 2.21b A
t = 30 days	3.37 ± 1.10b AB	0.97 ± 0.43b B	4.69 ± 1.27b A	5.25 ± 1.06 bc A
t = 60 days	4.01 ± 1.09b A	0.72 ± 0.25b B	3.99 ± 1.27b A	1.63 ± 0.45 cd B
t = 90 days	3.20 ± 0.40b A	0.47 ± 0.20b B	4.17 ± 1.33b A	0.48 ± 0.16 d B
LUGANA wine	**C**	**C - T4**	**KPA**	**KPA - T4**
t = 0 days	8.64 ± 0.22 a A	8.64 ± 0.22 a A	8.64 ± 0.22 a A	8.64 ± 0.22 a A
t = 10 days	7.62 ± 1.39 a A	1.53 ± 0.28b B	1.16 ± 0.27b B	1.10 ± 0.27b B
t = 30 days	1.88 ± 0.03b A	1.11 ± 0.25 bc B	0.88 ± 0.04b B	1.22 ± 0.30b B
t = 60 days	2.24 ± 0.61b A	1.02 ± 0.02 bc B	0.68 ± 0.16b B	0.93 ± 0.21b B
t = 90 days	1.53 ± 0.25b A	0.61 ± 0.12c B	0.73 ± 0.17b B	0.76 ± 0.14b B

Values with different capital letter indicate significant differences within lines ($p < 0.05$).
Values with different lowercase letter indicate significant differences within columns ($p < 0.05$).
* Each data represents the mean of three replicates ± standard deviation.

already 10 days after KPA addition and without refrigerated storage. Instead, the Cortese wine needed the KPA addition, refrigeration temperature, and 90 days of storage to reach the same turbidity level.

3.2. Effect on protein stability indices

Several protein stability tests are performed in the wine industry to define the dose of fining agent necessary in the wine stabilizing treatment concerning the protein haze formation (Cosme et al., 2020). The analytical tests normally applied include the determination of the total protein content or methods involving a reduction in wine protein solubility by heat-shock or chemicals (Pocock & Waters, 2006). Different precipitant agents can be used but they generate dissimilar precipitates and they do not perfectly reproduce the natural phenomenon. Therefore, the protein stability tests are not well correlate with total wine protein content. The wine protein fractions act differently and the protein assays are limited concerning the prediction of wine protein stability and the role of other wine components on protein instability is normally not considered (Pocock et al., 2007).

A valuable approach for research purposes can be to monitor and compare the experimental results of wine protein stability by different analytical methods. The results of Protein Charge Neutralization (PCN) test, Heat test (HT), and Cold Tannin Test (CTT) of untreated (C) and treated (KPA) wines, during the 90 days storage at ambient or refrigerated (T4) temperature, are reported in Table 2.

The Cortese wine showed a higher protein instability than Lugana, as indicated by PCN (10.25 ± 0.26 and 7.88 ± 0,92 respectively), HT (27.42 ± 1.24 and 5.22 ± 0.54), and CTT values (140.21 ± 4.68 and 65.87 ± 3.21). The control sample (C) for both wines showed the same protein instability along storage time when stored at ambient temperature, and no significant differences were pointed out by statistical analyses for PCN test. Instead, a slight decrease of HT and CTT results were observed after 60 days of storage. A lower storage temperature (T = +4°C) or polyaspartate addition (KPA) induced a significant decrease of protein stability indices, with higher decrease rate during storage. Moreover, it is possible to observe the combined effect of KPA addition and refrigeration temperature, especially for Cortese wine.

The potassium polyaspartate is negatively charged at wine pH and it can interact with haze-related proteins, that are positively charged, generating larger agglomerates that could precipitate faster over time

Table 2
Protein Charge Neutralization (PCN) test, Heat test (HT), and Cold Tannin Test (CTT) of untreated (C) and treated (KPA) samples of Cortese and Lugana wine, at different storage temperature (16 °C and 4 °C), and time (10, 30, 60, and 90 days).

	PROTEIN CHARGE NEUTRALIZATION (ΔNTU)				HEAT TEST (ΔNTU)				COLD TANNIN TEST (ΔNTU)			
CORTESE wine	C	C - T4	KPA	KPA - T4	C	C - T4	KPA	KPA - T4	C	C - T4	KPA	KPA - T4
t = 0 days	10.25 ± 0.56 a A*	10.25 ± 0.56 a A	10.25 ± 0.56 a A	10.25 ± 0.56 a A	27.42 ± 1.24 a A	27.42 ± 1.24 a A	27.42 ± 1.24 a A	27.42 ± 1.24 a A	140.21 ± 4.68 a A	140.21 ± 4.68 a A	140.21 ± 4.68 a A	140.21 ± 4.68 a A
t = 10 days	10.26 ± 0.16 a A	10.15 ± 0.52 a A	4.15 ± 0.15b B	3.66 ± 1.47b B	24.40 ± 0.10 a A	20.74 ± 1.94b B	15.22 ± 1.33b C	9.50 ± 1.43b D	138.42 ± 13.63 a A	73.09 ± 3.11b B	33.10 ± 2.47b C	7.47 ± 0.60b D
t = 30 days	10.94 ± 0.15 a A	8.03 ± 0.10b B	4.44 ± 0.96b C	3.12 ± 0.23 bc D	25.59 ± 0.81 a A	19.77 ± 0.52b B	13.96 ± 1.41b C	7.89 ± 1.76b D	137.68 ± 1.85 a A	74.81 ± 0.89b B	28.62 ± 1.47 bc C	5.06 ± 1.24b D
t = 60 days	9.95 ± 0.98 a A	6.62 ± 0.03c B	3.28 ± 0.60b C	1.97 ± 0.82 bc D	23.27 ± 4.01 a A	16.85 ± 1.93b B	7.31 ± 0.82c C	6.14 ± 1.13b C	119.07 ± 3.24b A	69.30 ± 5.95b B	19.33 ± 7.26c C	5.10 ± 0.86b D
t = 90 days	9.61 ± 0.14 a A	5.31 ± 0.20 d B	3.79 ± 0.36b C	1.28 ± 0.63c D	23.15 ± 2.90 a A	12.44 ± 1.65c B	6.57 ± 0.71c C	1.46 ± 0.46c D	68.44 ± 1.48c A	33.90 ± 7.00c B	17.77 ± 1.90c C	4.32 ± 1.13b D
LUGANA wine	C	C - T4	KPA	KPA - T4	C	C - T4	KPA	KPA - T4	C	C - T4	KPA	KPA - T4
t = 0 days	7.88 ± 0.92 a A	7.88 ± 0.92 a A	7.88 ± 0.92 a A	7.88 ± 0.92 a A	5.22 ± 0.54 a A	5.22 ± 0.54 a A	5.22 ± 0.54 a A	5.22 ± 0.54 a A	65.87 ± 3.21 a A	65.87 ± 3.21 a A	65.87 ± 3.21 a A	65.87 ± 3.21 a A
t = 10 days	7.15 ± 0.52 a A	6.74 ± 1.10 a A	3.92 ± 0.52b B	2.88 ± 0.71b B	4.49 ± 0.21 abc A	2.53 ± 0.42 bc B	1.15 ± 0.23b C	0.87 ± 0.06b C	65.01 ± 8.3 a A	55.64 ± 1.54b A	55.89 ± 5.72 bc A	62.53 ± 1.45 a A
t = 30 days	7.52 ± 0.08 a A	6.57 ± 0.37 a B	3.37 ± 0.10b C	2.93 ± 0.30b C	4.62 ± 0.45 ab A	3.01 ± 0.21b B	1.23 ± 0.09b C	0.92 ± 0.12b C	60.71 ± 2.12 a AB	52.44 ± 2.63 bc B	61.85 ± 2.81 ab AB	66.99 ± 5.84 a A
t = 60 days	8.46 ± 1.35 a A	8.34 ± 0.61 a A	3.38 ± 0.13b B	2.58 ± 0.30b B	3.53 ± 0.22c A	2.64 ± 0.11 bc B	1.02 ± 0.12b C	0.76 ± 0.23b C	57.90 ± 3.47 ab A	60.19 ± 4.59b A	58.91 ± 3.06 ab A	60.40 ± 0.93 a A
t = 90 days	7.91 ± 0.46 a A	6.57 ± 0.59 a B	2.81 ± 0.47b C	2.88 ± 0.33b C	3.72 ± 0.38 bc A	2.01 ± 0.19c B	0.98 ± 0.13b C	0.65 ± 0.13b C	46.93 ± 2.43b A	45.67 ± 4.50c A	50.54 ± 2.86c A	50.14 ± 1.57b A

Values with different capital letter indicate significant differences within line ($p < 0.05$).
Values with different lowercase letter indicate significant differences within column ($p < 0.05$).
* Each data represents the mean of three replicates ± standard deviation.

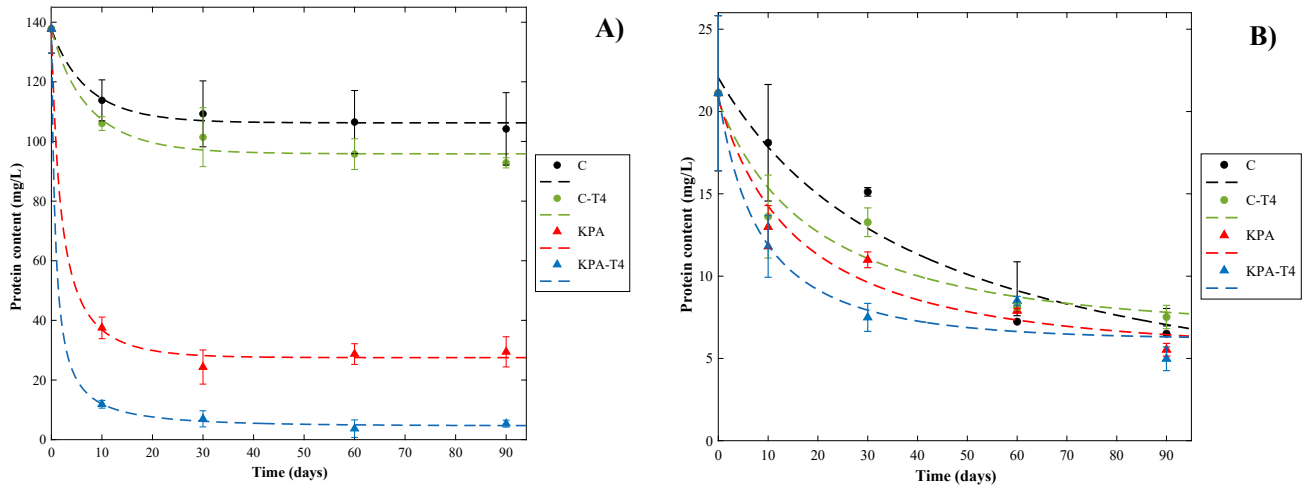

Fig. 1. Comparison between experimental mean values (●, ▲) and simulated ones (– – –) for total protein content (mg/L) of untreated (C) and treated (KPA) samples of Cortese (A) and Lugana (B) wine, at different storage temperature and time.

(Martínez-Pérez et al., 2020).

Instead, Lugana wine showed the same protein instability for control sample stored at both temperatures, but a significant decrease can be observed after KPA addition. It is notable that the cold tannin test highlighted a significant decrease of protein instability only after 90 days of storage. As yet reported, the protein stability tests are based on various precipitation agents and no correlated responses could be detected between the analytical assays.

3.3. Effect on unstable protein content (HPLC)

The HPLC analysis identified only thaumatin like proteins (TLPs) and no chitinases were found for both wines. Chitinases unfold at less temperature and they are sensitive to temperature variations and pH, both situations that normally occurs during the alcoholic fermentation. Instead, thaumatin-like proteins are characterized mainly by their higher thermostability and by presenting no significant conformational

Table 3
Relative areas of the main aroma classes of untreated (C) and treated (KPA) samples of Cortese wine, at different storage temperature (16 °C and 4 °C), and time (10, 30, 60, and 90 days).

Compounds	Sample	Time				
		t = 0	t = 10 days	t = 30 days	t = 60 days	t = 90 days
Aldheydes	C	0.35 ± 0.11 a B*	0.42 ± 0.02b AB	0.59 ± 0.14 a A	0.25 ± 0.04 bc B	0.21 ± 0.06 bc B
	C-T4	0.35 ± 0.11 a A	0.11 ± 0.01c B	0.14 ± 0.03b B	0.11 ± 0.01c B	0.11 ± 0.03c B
	KPA	0.35 ± 0.11 a AB	0.61 ± 0.05 a AB	0.63 ± 0.17 a A	0.31 ± 0.11b B	0.41 ± 0.10b AB
	KPA-T4	0.35 ± 0.11 a BC	0.16 ± 0.03c C	0.58 ± 0.10 a B	0.56 ± 0.06 a B	0.94 ± 0.18 a A
Fatty acids	C	52.53 ± 1.16 a A	55.24 ± 7.50 ab A	34.85 ± 0.78 a AB	29.86 ± 1.71b B	43.19 ± 7.82 a AB
	C-T4	52.53 ± 1.16 a A	67.58 ± 10.71 a A	56.81 ± 19.12 a A	65.28 ± 6.42 a A	52.15 ± 7.96 a A
	KPA	52.53 ± 1.16 a A	45.15 ± 7.87b A	45.32 ± 20.75 a A	39.77 ± 6.44 ab A	55.90 ± 7.52 a A
	KPA-T4	52.53 ± 1.16 a AB	66.22 ± 5.83 ab A	41.12 ± 7.34 a B	59.07 ± 10.63 a AB	48.84 ± 7.57 a AB
Alcohols	C	171.86 ± 1.73 a A	183.03 ± 29.28 a A	133.37 ± 2.63 a B	158.12 ± 6.11 a AB	147.93 ± 9.06 a AB
	C-T4	171.86 ± 1.73 a A	184.83 ± 24.25 a A	153.16 ± 39.96 a A	179.13 ± 11.68 a A	132.82 ± 7.31 a A
	KPA	171.86 ± 1.73 a A	219.80 ± 23.64 a A	173.26 ± 76.73 a A	160.59 ± 26.92 a A	132.83 ± 9.07 a A
	KPA-T4	171.86 ± 1.73 a A	197.07 ± 19.25 a A	127.00 ± 10.93 a B	168.47 ± 26.73 a AB	126.57 ± 9.23 a B
Ketones	C	0.06 ± 0.03 a A	0.14 ± 0.01 ab A	0.06 ± 0.02 a A	0.07 ± 0.02b A	0.08 ± 0.02b A
	C-T4	0.06 ± 0.03 a B	0.17 ± 0.03 a A	0.11 ± 0.02 a A	0.16 ± 0.02 a A	0.16 ± 0.04 a A
	KPA	0.06 ± 0.03 a A	0.11 ± 0.01b A	0.09 ± 0.04 a A	0.08 ± 0.01b A	0.10 ± 0.093 ab A
	KPA-T4	0.06 ± 0.03 a C	0.14 ± 0.01 ab A	0.08 ± 0.02 a BC	0.13 ± 0.03 a AB	0.15 ± 0.02 a A
Acetate esters	C	63.65 ± 0.96 a A	63.67 ± 0.35 ab A	51.56 ± 2.96 a A	57.11 ± 4.12b A	61.02 ± 9.33 a A
	C-T4	63.65 ± 0.96 a A	67.07 ± 1.43 a A	64.88 ± 11.33 a A	75.28 ± 5.93 a A	71.80 ± 7.10 a A
	KPA	63.65 ± 0.96 a A	58.48 ± 2.72b A	55.59 ± 19.14 a A	55.77 ± 7.01b A	64.64 ± 6.94 a A
	KPA-T4	63.65 ± 0.96 a A	61.10 ± 3.67 ab A	53.76 ± 4.30 a A	67.34 ± 7.89 ab A	75.44 ± 7.31 a A
Ethyl esters	C	578.25 ± 22.34 a A	600.97 ± 10.08 a A	584.02 ± 41.61 a A	525.67 ± 42.74 a A	609.54 ± 27.47 a A
	C-T4	578.25 ± 22.34 a A	501.64 ± 56.21 ab A	587.32 ± 47.21 a A	332.94 ± 156.84b AB	485.19 ± 21.18b B
	KPA	578.25 ± 22.34 a A	403.71 ± 28.84b B	405.57 ± 21.35b B	255.25 ± 9.09c D	328.60 ± 33.97c C
	KPA-T4	578.25 ± 22.34 a A	426.88 ± 46.90b B	294.64 ± 17.50c C	237.10 ± 54.32c C	323.07 ± 45.15c C
Other esters	C	5.82 ± 0.13 a A	6.08 ± 2.28 a A	5.44 ± 0.76 a A	4.51 ± 0.60 a A	4.97 ± 0.38 a A
	C-T4	5.82 ± 0.13 a A	6.16 ± 2.36 a A	5.51 ± 1.08 a A	3.57 ± 0.66 ab A	4.69 ± 1.20 a A
	KPA	5.82 ± 0.13 a A	3.33 ± 0.42 a B	2.21 ± 0.13b BC	2.14 ± 0.60b C	2.64 ± 0.47b BC
	KPA-T4	5.82 ± 0.13 a A	2.83 ± 0.39 a BC	2.38 ± 0.27b C	2.42 ± 0.56b C	3.74 ± 0.26 ab C
Terpenes	C	1.96 ± 0.08 a A	1.62 ± 0.04b B	1.21 ± 0.07 a C	1.20 ± 0.01b C	1.27 ± 0.20 a C
	C-T4	1.96 ± 0.08 a AB	2.19 ± 0.36 a A	1.39 ± 0.27 a B	1.64 ± 0.10 a B	1.52 ± 0.24 a B
	KPA	1.96 ± 0.08 a A	1.33 ± 0.11b A	1.35 ± 0.90 a A	1.22 ± 0.22b A	1.41 ± 0.14 a A
	KPA-T4	1.96 ± 0.08 a A	1.30 ± 0.09b B	1.02 ± 0.06 a B	1.36 ± 0.17 ab B	1.49 ± 0.06 a C

Values with different lowercase letter indicate significant differences within column ($p < 0.05$).
Values with different capital letter indicate significant differences within line ($p < 0.05$).
* Each data represents the mean of three replicates ± standard deviation.

variations or aggregation when exposed to pH variations (Cosme et al., 2020).

The initial protein content was significantly different between the two white wines, with a higher content for Cortese wine (137.86 ± 8.16 mg/L) than Lugana (21.12 ± 4.72 mg/L). The wine protein concentration can be associated with protein haze: wines with higher total protein concentration show also more predisposition to become unstable (Mesquita et al., 2001). A good linear correlation was found between protein concentration and PCN, HT, and CTT assays, with $R^2 > 0.85$ and R^2-adj > 0.84, only for Cortese wine. No linear correlation was highlighted for Lugana wine. As yet reported, the wine protein instability is not associated only to wine total protein content, but each individual wine protein fraction behaves differently and many other factors can affect the wine protein haze (Cosme et al., 2020). Fig. 1 shows the comparison between experimental mean values and simulated ones for total protein content (mg/L) of untreated (C) and treated (KPA) samples of Cortese (A) and Lugana (B) wine. The main effects of storage temperature and KPA addition were highlighted for Cortese wine, which showed a higher protein concentration and instability. The KPA addition induced a significant decrease of protein content already after 10 days, from 137.86 ± 8.16 mg/L to 37.49 ± 3.62 mg/L at ambient temperature. A combined effect of KPA addition and refrigerated temperature can be pointed out and a lowest protein content was achieved (11.85 ± 1.30 mg/L), corresponding to 92% decrease of the initial protein content. No significant differences can be highlighted at storage times longer than 10 days. Instead, the treatments of Lugana wine did not highlight the same results but a significant decrease of protein concentration was observed after the KPA addition and refrigerated storage. The sample KPA-T4 showed at 30 days the lowest protein content (8.49 ± 0.45 mg/L), corresponding to 60% of initial protein content. The KPA addition and refrigerated temperature induced an increase of protein precipitation, as depicted by the slope of the first part of simulated logistic model. At 90 days of storage, no significant differences can be observed between untreated (C and C-T4) and treated (KPA and KPA-T4) samples.

As depicted in Fig. 1, it is notable that the experimental data can be mathematical well described by the Logistic model. The criteria adopted to evaluate how well the empirical model represent the experimental data were the magnitudes of the coefficients of determination (R^2 and R^2-adj) and normalized root-mean-square deviation (NRMSD). Higher values of R^2 and R^2-adj and lower values of NRMSD denote a better goodness of fit and suggest that the model represents the experimental values well. The logistic function showed high accuracy and suitability for describing the protein removal capacity of KPA and its combined effect with temperature, as reported in **Table S1**.

The use of appropriate mathematical models can represent a valuable tool for optimizing the enological adjuvants and process time, especially from an economic point of view.

3.4. Effect on aroma composition

The use of enological adjuvants can affect the wine quality and aroma profile. The enological practices should preserve or better increase the quality of final product (Baiano et al., 2016; Silva-Barbieri et al., 2022). Therefore, a GC–MS analysis of Cortese and Lugana wines was carried out to detect some possible interactions of potassium polyaspartate on their aroma profile. The identified compounds were grouped in several categories (aldehydes, fatty acids, alcohols, ketones,

Table 4
Relative areas of the main aroma classes of untreated (C) and treated (KPA) samples of Lugana wine, at different storage temperature (16 °C and 4 °C), and time (10, 30, 60, and 90 days).

Compounds	Sample	Time				
		t = 0	t = 10 days	t = 30 days	t = 60 days	t = 90 days
Aldheydes	C	0.28 ± 0.03 a B*	0.78 ± 0.16 a A	0.81 ± 0.13 a A	0.67 ± 0.11 a A	0.66 ± 0.12 a A
	C-T4	0.28 ± 0.03 a B	0.22 ± 0.02b B	0.22 ± 0.05b B	0.32 ± 0.07b B	1.07 ± 0.27 a A
	KPA	0.28 ± 0.03 a C	0.53 ± 0.15 a B	0.75 ± 0.08 a AB	0.67 ± 0.09 a B	0.92 ± 0.05 a A
	KPA-T4	0.28 ± 0.03 a B	0.23 ± 0.02b B	0.16 ± 0.01b B	0.20 ± 0.03b B	0.94 ± 0.29 a A
Fatty acids	C	19.78 ± 1.36 a A	18.02 ± 1.93 a AB	18.13 ± 2.34 a AB	16.03 ± 0.27b AB	11.92 ± 5.52 a B
	C-T4	19.78 ± 1.36 a A	24.63 ± 7.07 a A	25.26 ± 3.56 a A	22.55 ± 1.14 a A	20.40 ± 4.90 a A
	KPA	19.78 ± 1.36 a A	21.73 ± 4.54 a A	20.91 ± 2.69 a A	17.05 ± 0.65 a A	14.19 ± 4.26 a A
	KPA-T4	19.78 ± 1.36 a A	26.93 ± 6.06 a A	19.75 ± 3.32 a A	17.50 ± 0.85 a A	17.78 ± 4.44 a A
Alcohols	C	96.47 ± 0.68 a A	84.96 ± 4.82 a A	83.28 ± 12.856 a A	81.80 ± 8.75b A	63.53 ± 37.59 a A
	C-T4	96.47 ± 0.68 a A	90.98 ± 15.79 a A	95.86 ± 18.26 a A	107.99 ± 3.01 a A	91.62 ± 18.43 a A
	KPA	96.47 ± 0.68 a A	84.47 ± 14.37 a A	85.56 ± 12.36 a A	82.17 ± 8.47b A	82.01 ± 17.57 a A
	KPA-T4	96.47 ± 0.68 a A	91.49 ± 18.62 a A	75.08 ± 6.42 a A	79.10 ± 6.80b A	106.89 ± 19.06 a A
Ketones	C	0.04 ± 0.01 a B	0.07 ± 0.01 a A	0.06 ± 0.01b AB	n.d.	n.d.
	C-T4	0.04 ± 0.01 a A	0.10 ± 0.04 a A	0.11 ± 0.02 a A	0.09 ± 0.01 a A	0.06 ± 0.06 a A
	KPA	0.04 ± 0.01 a A	0.06 ± 0.02 a A	0.06 ± 0.01b AB	0.00 ± 0.00b B	0.00 ± 0.00 a B
	KPA-T4	0.04 ± 0.01 a B	0.10 ± 0.02 a A	0.08 ± 0.01 ab A	0.08 ± 0.01 a AB	0.08 ± 0.02 a AB
Acetate esters	C	83.74 ± 4.28 a A	70.53 ± 1.10 a B	59.80 ± 8.64b BC	48.55 ± 2.26c CD	40.53 ± 2.71b D
	C-T4	83.74 ± 4.28 a A	88.48 ± 17.11 a A	95.15 ± 18.26 a A	102.18 ± 0.20 a A	80.64 ± 8.93 a A
	KPA	83.74 ± 4.28 a A	75.95 ± 11.05 a AB	61.23 ± 8.17b BC	49.87 ± 5.13c C	41.81 ± 8.22b C
	KPA-T4	83.74 ± 4.28 a AB	94.25 ± 9.76 a A	76.44 ± 1.88 ab B	79.18 ± 5.44b AB	78.06 ± 5.81 a B
Ethyl esters	C	387.59 ± 4.12 a A	381.62 ± 13.50 a A	307.54 ± 33.21b B	325.61 ± 11.58b B	312.82 ± 19.22 bc B
	C-T4	387.59 ± 4.12 a A	421.08 ± 80.43 a A	394.32 ± 35.12 a A	467.53 ± 4.83 a A	404.08 ± 36.79 a A
	KPA	387.59 ± 4.12 a A	327.64 ± 25.69 a A	228.06 ± 27.92c B	226.11 ± 17.97c B	238.28 ± 43.19c B
	KPA-T4	387.59 ± 4.12 a A	355.61 ± 47.31 a A	248.01 ± 16.53 bc A	316.29 ± 23.90b AB	327.60 ± 29.62 ab B
Other esters	C	3.85 ± 0.16 a A	4.10 ± 0.27 a A	3.55 ± 0.62 ab A	3.96 ± 0.04b A	3.81 ± 0.27 ab A
	C-T4	3.85 ± 0.16 a B	4.75 ± 1.18 a AB	4.60 ± 0.47 a AB	5.49 ± 0.13 a A	4.05 ± 0.40 a AB
	KPA	3.85 ± 0.16 a A	3.46 ± 0.47 a AB	2.52 ± 0.37b B	2.90 ± 0.28c B	2.65 ± 0.40b B
	KPA-T4	3.85 ± 0.16 a A	3.84 ± 0.45 a A	2.76 ± 0.48b A	3.44 ± 0.37 bc A	3.79 ± 0.65 ab A
Terpenes	C	0.89 ± 0.02 a A	0.91 ± 0.08 a A	0.72 ± 0.09 a A	0.65 ± 0.16 a A	0.67 ± 0.13 a A
	C-T4	0.89 ± 0.02 a A	1.02 ± 0.26 a A	0.97 ± 0.11 a A	0.84 ± 0.02 a A	0.71 ± 0.26 a A
	KPA	0.89 ± 0.02 a A	0.99 ± 0.15 a A	0.89 ± 0.15 a A	0.77 ± 0.07 a AB	0.56 ± 0.08 a B
	KPA-T4	0.89 ± 0.02 a A	0.98 ± 0.21 a A	0.78 ± 0.11 a A	0.62 ± 0.23 a A	0.70 ± 0.09 a A

Values with different lowercase letter indicate significant differences within column ($p < 0.05$).
Values with different capital letter indicate significant differences within line ($p < 0.05$).
* Each data represents the mean of three replicates ± standard deviation.

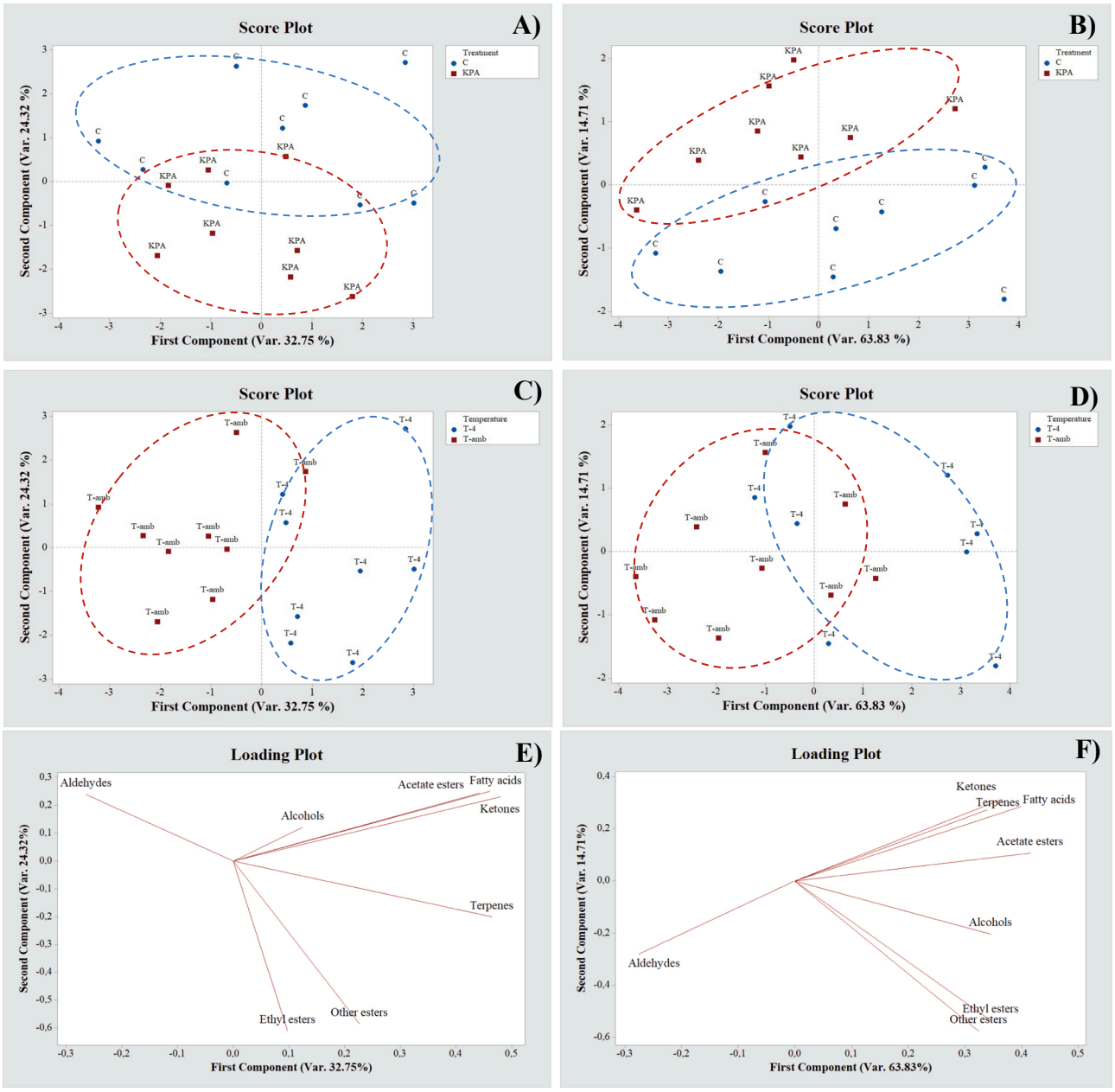

Fig. 2. Principal Component Analysis (PCA) results on relative areas of the volatile compounds detected in untreated and treated samples of Cortese (A, C, and E) and Lugana Wine (B, D, and F). Blue circles indicate the untreated wine (A,B) or wine stored at T-4 (C,D). Red circles indicate the treated wine with KPA (A,B) or wine stored at T-amb (C,D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acetate esters, ethyl esters, other esters, and terpenes), as reported in Table 3 and Table 4. The total sum of relative areas of aroma compounds was considered and subject to PCA. Fig. 2 shows the PCA results of Cortese (A, C) and Lugana samples (B, D). In Fig. 2**A and 2B** the samples can be discriminated into two groups based on the use of KPA addition, along the second component axis. The first component axis explains 32.8% and 63.8% of the total variance, while the second principal component explains 24.3% and 14.7% of the total variance for Cortese and Lugana wine, respectively. KPA addition for both wines affects the aroma content and a decrease trend of some chemical classes can be highlighted. For instance, independently of KPA addition and storage temperature, a mean decrease of ethyl esters, alcohols and superior alcohols was observed for Cortese and Lugana wine after polyaspartate addition (Fig. 2**E and 2F**). The decrease of aroma compounds has been also reported for other technological adjuvants, such as bentonite, which could interact directly with aroma compounds. Authors also demonstrated that part of the aroma compounds is removed through indirect mechanisms, because they are bound to the wine proteins (Vincenzi et al., 2015). Moreover, the different aroma categories and composition can show different interactions with wine colloids (Lubbers, Charpentier, et al., 1994; Lubbers et al., 2015; Lubbers, Voilley, et al., 1994).

It is notable that the separation of two groups based on KPA treatment is not well defined and a partial overlapping can be pointed out. Moreover, the total variance percentage of the second component is low for both wines and it means that PCA not explains well the experimental results.

Despite some significant changes on specific chemical compounds, overall, no statistical differences were pointed out between untreated (C) and treated samples for both wines. As reported in Table 4, the regression analysis based on total aroma concentration showed no significance for the treatment (X_1) and storage temperature (X_3). The KPA

Table 5
Regression analysis results of total aroma concentration for Cortese and Lugana wine.

Terms	Coefficients	
	CORTESE	LUGANA
Constant	874.7	592.8
Main Effects		
Treatment (X_1)	8.31	−4.03
Time (X_2)	−25.9***	−106.1***
Temperature (X_3)	12.57	48.31
Interaction effects		
$X_1 \cdot X_2$	−241***	−67.9
$X_1 \cdot X_3$	−13.53	−12.89
$X_2 \cdot X_3$	−65.25*	152.9***
$X_1 \cdot X_2 \cdot X_3$	−67.5	−64.4
R^2	85.0	92.4
R^2-adj	77.9	84.1

* $p < 0.05$; **$p < 0.01$;
*** $p < 0.001$.

addition, based on regression analysis, allowed the preservation of initial aroma concentration and no adversely effects were observed.

Fig. 2**C and 2D** shows that the Cortese and Lugana wines can be discriminated also into two groups based on the storage temperature. Acetate esters, terpenes, ketones, fatty acids, ethyl esters and alcohols are more abundant in refrigerated samples for both wines, instead aldehydes increase in both wines stored at ambient temperature (Fig. 2**E and 2F**). A low storage temperature decreases the volatility of aroma compounds and the acidic hydrolysis rate of acetic esters, preserving the wine sensorial properties (Espitia-Lopez et al., 2014; Pérez-Coello et al., 2003).

Significant differences can be pointed out on some aroma categories (Table 3 and 4). For instance, an increase of fatty acids, alcohols, acetate esters, ethyl esters, and terpenes was detected in Lugana wine when stored at 4 °C. An increase of fatty acids, acetate esters, and terpenes was detected also in Cortese wine.

Despite this, the PCA graph showed also a partial overlapping between the two groups discriminated by storage temperature. In view of the regression analysis on the total sum of aroma compounds, the storage temperature did not differentiate samples kept at ambient or refrigerated temperature.

Only the storage time resulted statistical significance ($p < 0.001$) and a general decrease of aroma concentration was observed, as depicted by the negative coefficients of the main effect of storage time (X_3). The statical analysis shows also significant some interaction effect between X_1 and X_3, or X_2 and X_3, as reported in Table 5.

4. Conclusions

Potassium polyaspartate, at the maximum concentration allowed by legislation, showed different effects for two white wines from northern Italy. The most valuable results were pointed out for Cortese wine, that showed higher initial turbidity, unstable protein concentration and instability.

The significant effects on wine turbidity indicated potential interaction between KPA and some wine macromolecules, affecting their sedimentation rates and wine clarification. The KPA addition positively affects the protein stability indices, particularly the Heat (HT) and Cold Tannin Test (CTT), achieving higher wine stability at 90 days of storage. The combined effect of temperature and KPA was also highlighted with higher decrease rate of protein stability indices during wine storage.

A good correlation was determined between the experimental results of stability indices and unstable protein concentration quantified by HPLC ($R^2 > 0.85$). The KPA addition and refrigerated temperature allowed a decrease of unstable protein up to 92% for Cortese wine. Mathematical model can be considered as a valuable tool for enological purposes, to optimize the stabilization practices and economic costs. The logistic function described well ($R^2 > 0.93$; NRMSD: 1.54–3.82%) the effect of KPA addition and temperature on the decrease of protein concentration along storage time.

Moreover, GC–MS analysis of Cortese and Lugana wines showed a decrease trend of some aroma compound classes, particularly in the first 10 days after KPA addition. Despite this, at the end of storage time no significant differences was detected between untreated and treated samples, for both wines. Potassium polyaspartate and storage temperature didn't adversely affect the aroma profile, as depicted also by PCA analysis. More experimental trials on several white wines, also evaluated by sensory analysis, are needed to understand better the effects of KPA addition on wine aroma and taste.

The potassium polyaspartate could be considered as a multifunctional enological adjuvant useful for tartaric and protein stability, without adversely effects on wine aroma profile. The potassium polyaspartate induce different effects on wine stability and quality, and it should be accurately managed according to wine variety and chemical composition.

CRediT authorship contribution statement

A. Natolino: Investigation, Data curation, Writing – original draft, Writing – review & editing. **L. Tat:** Formal analysis, Data curation, Writing – review & editing. **A. Gallo:** Data curation, Writing – review & editing. **T. Roman:** Validation, Writing – review & editing, Supervision. **E. Celotti:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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