Physico-Chemical Features of Sangiovese Wine as Affected by a Post-Fermentative Treatment with Chitosan

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Abstract: Chitosan is a natural biopolymer, which is gaining interest in red winemaking thanks to its ability to inhibit the development of Brettanomyces spp. yeast, or other undesired wine microbial threats. However, little is known about potential side-effects of its addition on the physico-chemical parameters of red wines. To fill the gap on this aspect, this work focused on changes in color, phenolic and volatile composition of red wines treated for 7 days with 0.5 g/L of fungoid chitosan, added in both undissolved and dissolved form. When compared to untreated samples, minor changes in phenolic compounds were observed in chitosan added wines, mainly involving hydroxycinnamic acids and flavonols, with reductions of 3 mg/L and 1.5 mg/L respectively. Ellagic acid, however, was absorbed up to 2 mg/L, which reduced his content by 40%. Since some of these compounds actively participate to co-pigmentation with anthocyanins, the color of wines was influenced accordingly. Chitosan marginally absorbed some aroma compounds, including volatile phenols whose amounts were slightly but significantly decreased after treatment. Overall, at the dose adopted, chitosan appeared suited to be used in red winemaking for microbial or physical stability purposes, not severely impairing the quality parameters of the final wines.

Keywords: chitosan; red wine; volatile phenols; color; co-pigmentation

1. Introduction

Chitosan is a biopolymer made of 2-Amino-2-deoxy-poly-p-glucosamine and N-acetyl glucosamine which is obtained from the deacetylation of chitin extracted from sources such as fungi, crustaceans, insects [1]. In the last decades, chitosan has aroused great interest in food industry due to its biocompatibility, biodegradability and low toxicity, being recognized as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA) [2]. In 2011, the use of chitosan from Aspergillus niger has been approved in oenology as an adjuvant for various purposes: clarification, reduction of heavy metal and toxins contents and for microbial stabilization [3]. Its insolubility in wine makes the removal by settling or filtration extremely easy. It has been demonstrated that chitosan treatments may lead to greater wine safety since it reduces the content of contaminating metals and ochratoxins [4]. Furthermore, chitosan is able to inhibit the growth of a wide range of spoilage microorganisms such as acetic acid bacteria, lactic acid bacteria and Brettanomyces spp. yeasts [5–7]. These latter are spoilage yeasts responsible for the production of volatile phenols (4-ethyl phenol and 4-ethyl guaiacol) and the consequent development of the “horse sweat”, “smoky” and “leather” character in wood aged or stored red wines, which are therefore severely compromised in quality. Already published results confirm that in red wines, the presence of 4–8 g/L chitosan for 6–10 days can effectively prevent the microbial developments, thus avoiding the risk of the mentioned sensory defect [5,8].
In this regard, it is also worth reporting that some authors have observed a reduction in the content of volatile phenols in the headspace of spoiled red wines treated with chitosan [9,10] which would suggest the potential for chitosan to remediate to adventitious development of Brettanomyces spp. in stored products. For these reasons, chitosan represents an interesting tool to manage the aging stage of red wines in wooden barrels, especially in the case of sulfite-free winemaking [11,12], where it may partially obviate the problems given by the absence of sulfur dioxide (SO\textsubscript{2}) [13].

However, little is known about the overall impact of chitosan treatment with respect to the fixed and volatile composition of red wines, taking into account the mode and the timing of addition typical for this adjuvant. A recent bibliographic review [13] tried to collate the scientific data available on this argument. It was claimed that at dose < 1 g/L chitosan seems not to significantly impact the color, the aroma, and the phenolic composition of red wines, but further investigation would be needed.

The aim of this work is, therefore, to evaluate the effect of chitosan applications on the chemical parameters of red wines, such as pH and titratable acidity and phenolic compounds especially phenolic acids, flavonols and anthocyanins and to deepen into the possible change in their chromatic features by evaluating co-pigmentation and polymers contribution the color. Additionally, the effect of the treatment on the volatile and sensory profile of wines previously contaminated with volatile phenols will be investigated. The application of two distinct addition modes (e.g., powder or dissolved chitosan) has also been compared with the purpose to evaluate the influence on the cited parameters of the increase in the specific surface of the polymer, since this parameter has been previously suggested to potentially impact the efficacy of the polymer in particular with respect to Brettanomyces spp. [7].

2. Materials and Methods

2.1. Chemicals and Chitosan Solutions

All chemicals were obtained from Sigma Aldrich (Sigma Aldrich, Milano). Fungal chitosan from Aspergillus Niger (80–90% deacetylated, Molecular weight: 10–30 KDa) was provided from Kitozyme (Hertal Belgium). A stock solution of dissolved chitosan (KTS) at 10 g/L was prepared in 10 g/L tartaric acid and kept in agitation overnight to facilitate dissolution.

2.2. Wine Samples

Red wines from Sangiovese grapes, vintage 2019, were obtained from local market. Sets of different trials of 100 mL wines were arranged in triplicate into 100 mL glass bottles by adding 0.5 g/L of pure chitosan in powder (KT) or 0.5 g/L of dissolved chitosan (KTS). A third set to be used for comparison purposes had no addition (C). Since the use of dissolved chitosan implied the contextual supplementation of 0.5 g/L of tartaric acid to KTS wines, an identical amount of tartaric acid was added to KT and C samples. Wines were agitated twice daily and after 7 days were centrifuged at 4500 \times g for 10 min at 20 °C, before analysis. Once added to the wine, solubilized chitosan (KTS) flocculated in few hours and precipitated after about 24 h, giving a fluffy deposit rather different from that obtained with powder chitosan (KT) which had quite a powdery appearance.

Subsequently, in order to further confirm the behavior of chitosan against volatile phenols, a parallel set of samples was prepared where treatments were performed on wines doped with 1500 µg/L 4-ethyl phenol (4-EP) and 500 µg/l 4-ethyl guaiacol (4-EG) to achieve values typical of heavily contaminated wines [14]. Experiments were performed identically as described before: C: control wine + ethyl phenols; KT: wine + 0.5 g/L of powder chitosan + ethyl phenols; KTS: wine + ethyl phenols + 0.5 g/L dissolved chitosan.

2.3. Oenological Parameters

General parameters were determined according to the International Organization of Vine and Wine (OIV) methods [15]. pH was measured by using a pH-meter (Mettler Toledo, Spain). Total phenolic
index (TPI) was assessed spectrophotometrically at 280 nm using a Jasco 810 spectrophotometer (Tokyo, Japan) and was expressed as mg/L of gallic acid (GAE). All the analyses were carried out by triplicate.

2.4. Analysis of Phenolic Compounds

Phenolic acid, flavanols and flavonols analysis [16] was carried out in a HPLC instrument equipped with a quaternary gradient pump Jasco PU-2089, an autosampler Jasco AS-2057 Plus Intelligent Sampler and two detectors: A Jasco UV/Vis MD-910 PDA detector and a Jasco FP-2020 Plus Fluorescence detector (Jasco, Tokyo, Japan). The column was a C18 Poroshell 120 (Agilent technologies, Santa Clara, CA, USA), 2.7µm, (4.6 × 150 mm), operating at 35 °C with a flow of 0.8 mL/min. Elution solvents were 2% acetic acid in HPLC grade water (Eluent A) and 2% acetic acid in HPLC grade acetonitrile (Eluent B). Gradient elution was as follows: from 98% to 95% A in 10 min, 95% to 90% A in 7 min, 90 to 82% A in 6 min, 82% to 80% A in 3 min, 80% to 70% A in 3 min, 70% to 50% A in 3 min, 50% to 0% A in 4 min and finally to 98% A in 1 min. Quantification was performed by means of calibration curves previously obtained by duplicate injections of pure standards solutions at known concentrations. All the analyses were carried out by triplicate. For compounds lacking pure standard, the calibration curve of structurally similar compound was used.

2.5. Chromatic Parameters

Color density and hue were determined by measuring optical densities at 420, 520 and 620 nm (1 cm of optical path) using a Jasco 810 spectrophotometer (Tokyo, Japan). CIELab parameters L* (Lightness), a* (redness), b* (yellowness), Cab* (chroma) and H(a* hue) were calculated according to OIV methods [15]. Percentage of color due to anthocyanins, co-pigmentation and polymerization was determined according to Boulton [17].

2.6. Identification and Quantification of Anthocyanins

Anthocyanins were analyzed by HPLC by the developed method of Chinnici and co-workers [18] using the HPLC instrument described before. Column: Synergi 4 µ Hydro-RP 80A (Phenomenex) 250 × 3. mm Elution program: initial conditions: (85% Solvent A (formic acid 10% in bidistilled water)/15% Solvent B (formic acid: acetonitrile: bidistilled water 10:45:45); 17 min 70%A/30%B; 45 min 27%A/73%B; 48 min 0%A/100%; 52 min 0%A/0%B/100%C (acetonitrile 100%); 55 min initial conditions 85%A/15%B. Quantification was carried out through a calibration curve of pure standard of malvidin-3-O-glucoside. All the analyses were carried out in triplicate.

2.7. Volatile Compounds

A method already described and validated by Lopez et al. [19] was used for volatile extraction. A hundred µL of a 2-octanol solution at 500 mg/L was added to 20 mL of wine as internal standard and deposed on a Lichrolut EN cartridge previously activated. Analytes were eluted with 5 mL of dichloromethane and concentrated to 200 µL under a stream of nitrogen prior to GC-MS analysis. The Trace Gas Chromatography (GC) ultra-apparatus coupled with a Trace DSQ mass selective detector (Thermo Fisher Scientific, Milan, Italy) was equipped with a fused silica capillary column Stabilwax DA (Restek, Bellefonte, PA, USA; 30 m, 0.25 mm i.d., and 0.25 µm film thickness). The carrier gas was He at a constant flow of 1.0 mL/min. The GC programmed temperature was: 45 °C (held for 3 min) to 100 °C (held for 1 min) at 3 °C/min, then to 240 °C (held for 10 min) at 5 °C/min. Splitless mode injection (1 µL) was performed at 250 °C. Detection was carried out by electron ionization (EI) mass spectrometry in full scan mode, using ionization energy of 70 eV. Transfer line interface was set at 220 °C and ion source at 260 °C. Mass acquisition range was m/z 30–400. Compounds were identified by a triple criterion: (i) by comparing their mass spectra and retention time with those of authentic standards, (ii) compounds lacking of standards were identified after matching their respective mass spectra with those present in the commercial libraries NIST 08 and Wiley 7, (iii) matching the linear retention index (LRI) obtained under our conditions, with already published LRI on comparable
polar columns. Quantification of compounds was carried out via the respective total ion current peak areas after normalization with the area of the internal standard. Calibration curves were obtained by injections of standard solutions, subjected to the cited extraction procedure, containing a mixture of commercial standard compounds at concentrations between 0.01 and 200 mg/L, and internal standard at the same concentration as in the samples. The calibration equations for each compound were obtained by plotting the peak area response ratio (target compound/internal standard) versus the corresponding concentration. For compounds lacking reference standards, the calibration curves of standards with similar chemical structure were used. Analyses were done in triplicate.

2.8. Identification of Phenolics Absorbed onto Chitosan

After 7 days of treatment and centrifugation of samples, chitosan remaining in the bottom of centrifuge tube was extensively washed with distilled water and filtered under vacuum with 0.2 µm polyamide filters (Sartorius, Goettingen, Germany). Successively, chitosan was dissolved in 10 mL of pure formic acid, centrifuged and the surnatant, containing the absorbed phenolics, was dried in a rotary evaporator. Pellet was dissolved in 5 mL of a 50% H2O/methanol solution. Absorbed phenolic compounds were analyzed by following the HPLC method described in Section 2.6.

2.9. Sensory Analysis

Sensory analysis was performed by 22 (10 men and 12 women aged from 27 to 52) well-trained panelists recruited from the staff of the Department of Agricultural and Food Sciences of the University of Bologna. Tasting cards were developed by our research group for red wines spiked with ethyl phenols in order to assess appearance and aromatic profile of samples treated with solid (KT) and dissolved (KTS) chitosan. A total of 9 sensory descriptors were selected, 2 for appearance (color intensity and limpidity) and 7 for the aroma (fruity, floral, greenness, smokey, animal, rubber and aromatic intensity). A preliminary triangular test was performed followed by a quantitative descriptive analysis (QDA) on a structured scale from 1 (absent) to 5 (maximum). All sessions were performed in a normalized room according to ISO 8589:2007. Samples were arranged in coded and capped wine glasses (ISO 3591:1977). Statistical analysis was performed by one-way analysis of variance (ANOVA).

2.10. Statistical Treatment

Statistical analysis of dataset was performed by means of the XLSTAT Software package Version 2013.2 (Addinsoft, Paris, France). One-way analysis of variance (ANOVA) followed by a post hoc comparison (Tukey’s HSD test) was carried out.

3. Results and Discussion

3.1. General Parameters

3.1.1. pH and Total Acidity

As illustrated in Table 1, all general parameters were influenced by the presence of chitosan when compared to the control (C). pH was increased after the treatment with chitosan (KT) or with dissolved chitosan (KTS) (0.05 and 0.06 units respectively). As already reported by others [20,21], this trend may be due to a reduction in the content of organic acids absorbed by chitosan through an electrostatic interaction between its charged amino groups (-NH3+) and the anionic form of organic acids present in wine (Reactions 1–3):

\[
\text{CHIT-NH}_2 \leftrightarrow \text{CHIT-NH}_3^+ \quad (1)
\]

\[
\text{AH} + \text{H}_2\text{O} \leftrightarrow \text{A}^- + \text{H}_3\text{O} \quad (2)
\]

\[
\text{CHIT-NH}_2 + \text{AH} \leftrightarrow \text{CHIT-NH}_3^+ \text{A}^- \quad (3)
\]

\[
\text{CHIT-NH}_2 + \text{H}_2\text{O} \leftrightarrow \text{CHIT-NH}_3^+ + \text{OH}^- \quad (4)
\]
Table 1. Oenological parameters of red wines after 7 days of treatments. In the same row, different letters indicate significant differences according to Tukey’s test ($p < 0.05$). $n = 3$. C: no addition; KT: 0.5 g/L of solid chitosan; KTS: 0.5 g/L of chitosan in solution. TPI: total phenolic index. GAE: gallic acid equivalent. AU: absorbance units.

|                     | C            | KT           | KTS          |
|---------------------|--------------|--------------|--------------|
| pH                  | 3.65 ± 0.01  | 3.70 ± 0.01  | 3.71 ± 0.01  |
| Titratable acidity (g/L) | 5.89 ± 0.13  | 5.61 ± 0.09  | 5.74 ± 0.01  |
| TPI (GAE) (mg/L)    | 1286 ± 17.43 | 1179 ± 19.23 | 1170 ± 4.14  |
| Abs 420 nm (AU)     | 1.567 ± 0.02 | 1.423 ± 0.01 | 1.421 ± 0.01 |
| Abs 520 nm (AU)     | 1.690 ± 0.02 | 1.528 ± 0.01 | 1.528 ± 0.01 |
| Abs 620 nm (AU)     | 0.455 ± 0.02 | 0.376 ± 0.03 | 0.337 ± 0.04 |
| Hue                 | 0.939 ± 0.01 | 0.931 ± 0.01 | 0.930 ± 0.01 |
| Color density (AU)  | 3.730 ± 0.04 | 3.327 ± 0.01 | 3.386 ± 0.02 |

Accordingly, samples added of solid chitosan also resulted lower in titratable acidity (Table 1). However, when dissolved, chitosan tended to behave somehow differently, suggesting that acidity could be influenced by a further mechanism. For example, as already proposed by Gyliene and co-workers [22], chitosan amino groups could get positively charged once in aqueous solution by hydrogen abstraction from water, leading to the generation of hydroxyl anions (OH-) and the consequent rise in pH (reaction 4). The role of these two mechanisms as affected by the addition mode of chitosan (powder or dissolved) remains to be investigated.

However, if the weaker impact of dissolved chitosan on titratable acidity is confirmed by further studies, this addition mode could be an interesting alternative to solid form in order to minimize the effect of this polysaccharide on the acidic parameters of white wines.

3.1.2. Total Phenolics

As displayed in Table 1, both treatments with chitosan drove to a significant reduction of total phenolic index of wines. Other authors also observed a diminution of this parameter after addition of 1 g/L chitosan in white or model wines [23–25] due to absorption onto the polymer surface. Data reported by Milheiro et al. [10] and Filipe-Ribeiro et al. [9] after treating red wines with different chitosans at 0.1 g/L revealed no differences when compared to untreated samples. However, doses of 1 g/L did induce about 10% reduction of the TPI parameter, percentage slightly higher to the one we obtained in the present study. The above results, together with HPLC analysis of phenolic acids will be further discussed in the next section.

3.1.3. Abs 420, 520, 620 nm

To evaluate the effect of chitosan on the color of red wines, in a first approach the optical density at 420, 520 and 620 nm was measured (Table 1). As also already described elsewhere [23,26], chitosan addition resulted in a reduction of the absorbance related to yellow nuances (420 nm) regardless the physical form of addition (dissolved or solid). Lower values of optical densities at 520 and 620 nm on KT and KTS were also recorded. This fact may be the result of a diminished content of anthocyanins and polymers, responsible for the reddish and bluish pigmentation. Consequently, hue and color density were also decreased after the addition of chitosan. The compounds that actively participate to the color of the wines, that is phenolic acids, flavonols and anthocyanins, have been analyzed and discussed in the following sections.

3.2. Phenolic Acids

HPLC analysis of phenolic acids after 7 days of treatment is reported in Table 2. If compared to the control, some compounds belonging to the classes of hydroxycinnamic acids, benzoic acids and flavonols were slightly reduced in quantity after treatment, without remarkable differences between solved (KTS) or solid chitosan (KT). As also mentioned previously, the presence of different functional groups in the chitosan backbone such as hydroxy (-OH), amino (-NH$_2$) and acetamido (-CONH$_2$) allow
the absorption of phenols through weak interactions such as hydrogen bonding and van der Waals forces [23–25]. Moreover, at wine pH, phenolic acids are present in their dissociated phenolate anionic form, and may therefore, be absorbed onto chitosan surface by electrostatic interaction with protonate amino groups [25].

Table 2. Phenolic acids, flavonoids and other phenolics identified and quantified (mg/L) in red wines after 7 days of treatment. In the same row, different letters indicate significant differences according to Tukey’s test (p < 0.05). n = 3. C: no addition; KT: 0.5 g/L of solid chitosan; KTS: 0.5 g/L of chitosan in solution. GRP: grape reaction product.

| Phenol                     | C       | KT      | KTS     |
|----------------------------|---------|---------|---------|
| **Flavanols**              |         |         |         |
| Procyanidin B1             | 8.46 ± 0.25 a | 8.31 ± 0.16 a | 8.37 ± 0.23 a |
| Catechin                   | 25.52 ± 1.34 a | 25.14 ± 1.15 a | 25.33 ± 0.97 a |
| Procyanidin B3             | 3.50 ± 0.10 a | 3.44 ± 0.06 a | 3.50 ± 0.10 a |
| Procyanidin B2             | 7.10 ± 0.43 a | 7.02 ± 0.37 a | 7.05 ± 0.27 a |
| Epicatechin                | 17.57 ± 1.03 a | 17.35 ± 0.89 a | 17.40 ± 0.69 a |
| **Sum flavanols**          | 62.15 ± 3.15 a | 61.27 ± 2.61 a | 61.64 ± 2.24 a |
| **Benzolate derivatives**  |         |         |         |
| Gallic Acid                | 38.21 ± 0.85 a | 37.71 ± 0.21 a | 38.05 ± 0.47 a |
| Ethyl gallate              | 8.28 ± 0.33 a | 8.29 ± 0.19 a | 8.32 ± 0.07 a |
| Protocatechuic acid        | 7.97 ± 0.10 a | 8.01 ± 0.26 a | 7.96 ± 0.17 a |
| Ellagic acid               | 5.27 ± 0.06 a | 3.02 ± 0.02 b | 3.17 ± 0.03 b |
| **Sum benzoates**          | 59.73 ± 0.74 a | 57.03 ± 0.56 a | 57.5 ± 0.24 a |
| **Hydroxycinnamates derivatives** |     |         |         |
| cis-Coumaric               | 2.91 ± 0.07 a | 2.91 ± 0.02 a | 2.97 ± 0.01 a |
| trans-Coumaric             | 7.57 ± 0.14 a | 7.49 ± 0.03 a | 7.55 ± 0.01 a |
| p-Coumaric acid            | 1.97 ± 0.14 a | 1.71 ± 0.02 b | 1.71 ± 0.02 b |
| cis-Caftaric acid          | 2.36 ± 0.02 a | 2.21 ± 0.04 a | 2.21 ± 0.02 a |
| trans-Caftaric acid        | 24.54 ± 0.44 a | 23.32 ± 0.11 b | 23.72 ± 0.17 b |
| GRP                        | 5.44 ± 0.05 a | 4.55 ± 0.04 c | 4.63 ± 0.02 b |
| trans-Ferulic acid         | 2.08 ± 0.02 a | 2.04 ± 0.03 a | 2.05 ± 0.01 a |
| trans-Caffeic acid         | 5.07 ± 0.32 a | 4.30 ± 0.01 b | 4.31 ± 0.02 b |
| **Sum hydroxycinnamates**  | 49.56 ± 0.96 a | 46.27 ± 0.22 b | 46.94 ± 0.24 b |
| **Flavonols**              |         |         |         |
| Flavonol derivative        | 1.23 ± 0.01 a | 1.23 ± 0.01 a | 1.24 ± 0.01 a |
| Dihydromyricetin           | 1.41 ± 0.01 a | 1.39 ± 0.01 a | 1.40 ± 0.02 a |
| 3-O-rhamnoside             | 1.87 ± 0.06 a | 1.76 ± 0.04 a | 1.78 ± 0.05 a |
| Myricetin glucuronide      | 4.24 ± 0.15 a | 4.09 ± 0.08 a | 4.12 ± 0.11 a |
| Myricetin 3-glucoside      | 1.97 ± 0.07 a | 1.91 ± 0.02 a | 1.92 ± 0.02 a |
| Quercetin glucuronide      | 5.95 ± 0.10 a | 5.68 ± 0.03 b | 5.75 ± 0.02 b |
| Quercetin 3-glucoside      | 4.55 ± 0.09 a | 4.46 ± 0.04 a | 4.52 ± 0.02 a |
| Syringetin 3-glucoside     | 1.57 ± 0.03 a | 1.57 ± 0.02 a | 1.57 ± 0.00 a |
| Myricetin                  | 2.27 ± 0.09 a | 2.13 ± 0.07 b | 2.21 ± 0.04 ab|
| Quercetin                  | 6.90 ± 0.28 a | 6.23 ± 0.14 b | 6.40 ± 0.36 ab|
| **Sum flavonols**          | 31.97 ± 0.25 a | 30.46 ± 0.08 c | 30.91 ± 0.16 b |
| **Stilbenes**              |         |         |         |
| Resveratrol glucoside      | 0.89 ± 0.01 a | 0.89 ± 0.00 a | 0.89 ± 0.00 a |
| t-Resveratrol              | 1.03 ± 0.01 a | 1.02 ± 0.00 a | 1.03 ± 0.01 a |
| **Others**                 |         |         |         |
| Tirosol                    | 27.59 ± 0.71 a | 27.36 ± 0.32 a | 27.58 ± 0.34 a |

Our results revealed that the presence of chitosan affected to a greater extent the amount of hydroxy cinnamic acids by reducing preferentially the concentration of caffeic acid derivatives (caftaric acid, GRP and caffeic acid) and coumaric acid, reaching removal levels up to 1.22 mg/L and 0.89 mg/L for caftaric acid and GRP respectively (Table 2). The interaction of chitosan with this class of compounds was found by Spagna et al. [25] to follow a typical “cooperative adsorption” scheme where hydroxycinnamates could set up forces with molecules already adsorbed on the polysaccharide surface. In particular, the mechanism involves the electrostatic interaction between carboxylic groups of hydroxycinnamates and the protonate amine groups of chitosan which is facilitated by a perpendicular
orientation of this acid on the polymer surface. Such adsorbed molecules may further associate side by side through the formation of π-π bonds among their aromatic rings. This trend may be of interest in the case of treatments of red wines aiming the control of *Brettanomyces* spp. since hydroxycinnamic acids are the precursors of volatile phenols, the main compounds responsible of the negative aroma of wines contaminated by those yeasts [27].

Even if to a lesser extent, also flavonols diminished in wines after treatment (up to 1.50 mg/L reduction as a sum). As outlined in Table 2, concentrations of quercetin glucuronide, myricetin and quercetin were slightly reduced by the presence of both chitosan formulations, being KT apparently more impacting than KTS. de Oliveira and co-workers studied the interaction between flavonols and chitosan by means of molecular modeling [28]. They showed the existence of a stable attractive interaction with quercetin and quercitrin due to the planarity of those molecules which favors the setting up of hydrogen bonds with the polar groups of chitosan. A similar mechanism could also be evoked for the diminution of ellagic acid (Table 2), another planar and extensively hydroxylated molecule. It is worth noting that all these compounds could participate to the “cooperative absorption” described in the above paragraph, since their absorption onto chitosan may stabilize hydroxycinnamates on its surface thanks to π-π stacking involving the aromatic rings of such molecules. Overall, these data explain the lower TPI of KT and KTS samples reported in Table 1. Together with anthocyanins, flavonols and cinnamates may also influence the color expression of wines, which will be evaluated in the following section.

### 3.3. Effect of Chitosan on Color Features and Anthocyanins Composition of Wine

CIELab parameters, co-pigmentation magnitude and anthocyanins content of samples were evaluated and presented in Tables 3 and 4. Both chitosan treatments (KT and KTS) reduced significantly the contribution of co-pigmentation and polymerization to the color of wines. Co-pigmentation is a phenomenon of reinforcement of wine color favored by the presence of π conjugated systems and hydrogen bond donor/acceptor groups of certain molecules (co-pigments) which leads to the formation of π-π stacking interactions with anthocyanins [29]. In red wines, the main compounds described as powerful copigments are (i) flavonols such as quercetin or myricetin, due to the extended conjugation of the three constitutive aromatic rings and (ii) hydroxycinnamic acids, especially the ones closely linked to anthocyanins by acylation of glycosyl groups [29,30]. Interestingly, as previously discussed, the content of these two families of compounds, were particularly reduced after chitosan treatments.

| Table 3. Percentage contribution to the color and CIELab parameters of wines after 7 days of treatment with chitosan. In the same row, different letters indicate significant differences according to Tukey’s test ($p < 0.05$). $n = 3$. C: no addition; KT: 0.5 g/L of solid chitosan; KTS: 0.5 g/L of chitosan in solution. |
|---|---|---|
| **CIELab parameters** | **C** | **KT** | **KTS** |
| L* | 19.43 ± 2.62 b | 25.72 ± 0.11 a | 24.1 ± 0.51 a |
| a* | 48.17 ± 3.28 b | 54.82 ± 0.14 a | 52.8 ± 0.43 a |
| b* | 34.98 ± 4.03 b | 40.10 ± 0.08 a | 37.9 ± 0.55 a |
| C* | 57.83 ± 4.97 b | 67.93 ± 0.16 a | 65.0 ± 0.67 a |
| h<sub>ab</sub> | 33.50 ± 1.49 b | 36.19 ± 0.05 a | 36.7 ± 0.18 a |
| **Co-Pigmentation** | 9.7 ± 2.7 a | 0.0 ± 0.1 c | 6.1 ± 1.8 b |
| **% Anthocyanins** | 36.5 ± 2.1 b | 52.5 ± 4.0 a | 46.9 ± 2.1 a |
| **% Polymers** | 53.8 ± 0.7 a | 47.5 ± 2.4 b | 47.0 ± 0.8 b |
As a general trend, co-pigmentation and polymerization observed in chitosan-treated samples (Table 3) corresponded to increased luminance (L*) and increased yellowness (b*). These results are in line with the lower color intensity found for KT and KTS (Table 1). Judging by our results on co-pigment composition, a lower chroma (C*) would be expected. Surprisingly, treatments with chitosan exhibited higher C* and b* values, i.e., increased

Besides, a particular attention should be paid to ellagic acid since this compound, because of its extended planar conformation, has demonstrated to spontaneously binds to pigments by means of Van der Waals interactions, conferring to this molecule a high co-pigmentating potential [31,32].

Thus, the reduced co-pigmentation levels in samples treated with chitosan could be due to a lesser content of available cofactors already adsorbed on chitosan, e.g., hydroxycinnamic acids, flavonols and ellagic acid.

In Table 3, a lower percentage of color due to polymers is also shown for chitosan samples. The reason to this evidence may be partially due to the reduction of vitisins or ethyl adducts which will be commented in the following section. Those compounds, in fact, being stable to SO₂ addition, are included into the polymeric fraction by the analytical method we used.

CIELab parameters significantly changed after treatments with chitosan (Table 3) in accordance with what already found in a previous research [9]. As a general trend, co-pigmentation and self-association have been shown to produce a hyperchromic effect on red wines, leading them to darker colors (lower L*), and increased saturation (C*), also depending on the co-factor considered [33–35].

The above statement partly coincides with our results, where reduced co-pigmentation and polymerization observed in chitosan-treated samples (Table 3) corresponded to increased luminance (L*) and increased yellowness (b*). These results are in line with the lower color intensity found for KT and KTS (Table 1). Judging by our results on co-pigment composition, a lower chroma (C*) would be expected. Surprisingly, treatments with chitosan exhibited higher C* and b* values, i.e., increased

| Table 4. Anthocyanins content of wines after 7 days of treatment with chitosan. In the same row, different letters indicate significant differences according to Tukey’s test (p < 0.05). n = 3. C: no addition; KT: 0.5 g/L of solid chitosan; KTS: 0.5 g/L of chitosan in solution. |
|---|---|---|
| | C | KT | KTS |
|**Glucosides** | | | |
| Di-3-glc | 6.05 ± 0.07 a | 6.14 ± 0.08 a | 5.95 ± 0.24 a |
| Ch-3-glc | 2.65 ± 0.14 a | 2.61 ± 0.03 a | 2.44 ± 0.23 a |
| Pt-3-glc | 10.06 ± 0.25 a | 9.99 ± 0.06 a | 9.96 ± 0.13 a |
| Mn-3-glc | 6.22 ± 0.14 a | 6.08 ± 0.06 a | 6.08 ± 0.08 a |
| Mv-3-glc | 39.49 ± 1.32 a | 39.75 ± 0.37 a | 39.79 ± 0.60 a |
| total glc | 64.47 ± 1.90 a | 64.57 ± 0.54 a | 64.22 ± 0.68 a |
|**Acetylglucosides** | | | |
| Di-3-acetylglc | 0.22 ± 0.00 a | 0.22 ± 0.01 a | 0.23 ± 0.01 a |
| Pt-3-acetylglc | 0.18 ± 0.02 a | 0.19 ± 0.01 a | 0.17 ± 0.05 a |
| Mn-3-acetylglc | 0.56 ± 0.02 a | 0.56 ± 0.01 a | 0.56 ± 0.02 a |
| Mv-3-coumaroylglc | 0.42 ± 0.01 a | 0.42 ± 0.01 a | 0.42 ± 0.02 a |
| total acetglc | 1.39 ± 0.05 a | 1.40 ± 0.02 a | 1.38 ± 0.05 a |
|**Ethyl-bridged adducts** | | | |
| Mv-3-glc (epi) catechin | 0.77 ± 0.05 a | 0.75 ± 0.01 a | 0.76 ± 0.02 a |
| Mn-3-glc-ethyl-(epi)-catechin | 0.60 ± 0.02 a | 0.59 ± 0.01 a | 0.58 ± 0.02 a |
| Mv-3-glc-ethyl-(epi)-catechin | 1.05 ± 0.01 a | 1.04 ± 0.01 a | 1.05 ± 0.02 a |
| Mv-3-glc-ethyl-(epi)-catechin | 0.77 ± 0.02 a | 0.76 ± 0.03 a | 0.78 ± 0.01 a |
| total ethyl ep (cat) | 3.18 ± 0.06 a | 3.13 ± 0.02 a | 3.16 ± 0.03 a |
|**B-type vitisins** | | | |
| B-type vitisin of Pt-3-glc | 0.19 ± 0.02 a | 0.17 ± 0.01 a | 0.18 ± 0.01 a |
| B-type vitisin of Mn-3-glc | 0.20 ± 0.01 a | 0.20 ± 0.01 a | 0.22 ± 0.01 a |
| B-type vitisin of Mn-3-glc (vitisin B) | 0.95 ± 0.08 a | 0.91 ± 0.01 a | 0.93 ± 0.04 a |
| total B-type | 1.35 ± 0.09 a | 1.28 ± 0.03 a | 1.32 ± 0.04 a |
|**A-type vitisins** | | | |
| A-type vitisin of Mv-3-glc (vitisin A) | 1.02 ± 0.03 a | 0.99 ± 0.01 ab | 0.97 ± 0.01 b |
| A-type vitisin of Pt-3-glc | 0.72 ± 0.02 a | 0.67 ± 0.00 b | 0.70 ± 0.01 a |
| total A-type | 1.75 ± 0.01 a | 1.66 ± 0.01 b | 1.67 ± 0.02 b |
|**Vinylphenol adducts** | | | |
| Mv-3-glc-4-vinyl (epi) catechin | 0.44 ± 0.02 a | 0.40 ± 0.00 b | 0.38 ± 0.01 b |
| Pinotin A | 0.24 ± 0.04 a | 0.24 ± 0.02 a | 0.22 ± 0.02 a |
| Mn-3-glc-4-vinylphenol | 0.14 ± 0.02 a | 0.11 ± 0.00 b | 0.13 ± 0.01 ab |
| Mv-3-glc-4-vinylphenol | 0.82 ± 0.06 a | 0.78 ± 0.02 a | 0.82 ± 0.03 a |
| Mv-3-glc-4-vinylflavonol | 0.14 ± 0.02 a | 0.11 ± 0.01 a | 0.13 ± 0.02 a |
| Total vinyl | 1.77 ± 0.11 a | 1.65 ± 0.05 a | 1.68 ± 0.07 a |
| Total anthocyanins | 149.28 ± 3.95 a | 148.72 ± 1.09 a | 148.27 ± 1.52 a |
red color vividness as reported on Table 3. This also depended on the removal by chitosan of some specific pigments as discussed below. Color perception of samples was compared by calculating ∆E*ab, where ∆E*ab > 3 indicates differences visible to the human eye [36]. Our experiments showed a value of ∆E*ab of 11.93 when comparing C and KT, 8.5 between C and KTS and 3.49 for KT vs KTS, demonstrating that all samples were visually distinguishable from each other, especially between chitosan treatments and the control.

Samples anthocyanins amount are showed in Table 4. In general, monomeric anthocyanins seemed not to be influenced by the presence of chitosan, as previously reported by some authors who used up to 1 g/L of chitosan [9,10]. Vitisin B were a further class of compounds not affected by the treatment while vitisin A did demonstrate to be marginally but significantly lower in KT wines. Synthesis of vitisin A occurs through the reaction between malvidin-3-O-glucoside and pyruvic acid. At wine pH, this molecule is present in a mixture of neutral and anionic forms [37] that would facilitate its interaction with positively charged amine groups of chitosan. Together with the extended aromatic conjugation of vitisin A, such interaction may further stabilize adsorption onto chitosan surface which is in consonance to the outlined reduced co-pigmentation (Table 3). Slightly differences were also observed for hydroxyphenyl pyranoanthocyanins, particularly Mv-3-glc-4-vinyl-(epi)-catechin and Pn-3-glc-4-vinylphenol whose amounts were lower in samples treated with chitosan (Table 4). Together with vitisin A, these compounds contribute to the orange-yellowish nuance of red wines and their diminution, albeit modest, could be the reason for lower hue (Table 1), higher hab and red color vividness (Table 3) recorded for such samples.

To give a general and qualitative view about the type of molecules adsorbed onto chitosan, one of the formulations (KTS, in particular) was removed from wine after fining and the adsorbed compounds extracted following polymer acid dissolution. Figure 1 shows a chromatogram of the identified compounds. As depicted, the main peaks were attributed to hydroxycinnamic acids, ellagic acid and quercetin while only traces of anthocyanins were observed, which perfectly match with the data coming from wine analysis (Table 2). Despite the adopted collection and extraction procedures did not permit to perform the accurate quantification of the molecules released from chitosan, a comparison with identical chromatograms from wine samples (not shown) allowed to evaluate the reciprocal affinity of these compounds with respect to the adjuvant. In this regard, ellagic and caftaric acids were the phenolics whose peak area ratio after/before the treatment increased the most, suggesting the highest affinity with chitosan. Furthermore, the presence onto the polymers surface of the identified molecules demonstrates that their diminution in wine samples is unlikely to be due to oxidation or other chemical modifications artificially induced by chitosan.

3.4. Volatile Profile

Figure 2A shows the total amount of the different volatile compounds grouped by chemical families. As can be seen, neither KT nor KTS appeared to significantly affect the overall concentrations of the main classes of compounds.

When individually considered, however, among alcohols, slight but significant impacts were found on n-butanol and n-hexanol contents due to KT and KTS respectively (Table A1). For n-hexanol, previous results reported analogous reductions after fining with solid chitosan [38,39] added at higher doses (1 g/L). Solubilization of chitosan (KTS) may be hypothesized to increase specific surface, leading to a higher absorption capacity.

Regarding esters, it is noteworthy that both chitosan treatments resulted in a lower content of ethyl cinnamate (Appendix A, Table A1). This trend appears to be directly correlated to the results of Section 3.2. Structural similarity between ethyl cinnamate and cinnamic acid leads to analogous absorption of the ester onto chitosan surface giving rise to the decrease of its concentration in the wine.

According with previous studies [39,40] furanic compounds, mainly coming from Maillard and Strecker reactions between sugars and amino acids, were found at higher concentrations after chitosan treatments (Figure 2A). This trend, as already investigated by Nunes and co-workers [40], is correlated
to the presence of amine groups on chitosan backbone that could promote Maillard and Strecker reactions by reacting with wine metabolites such as residual sugars even if, according to those authors, the formation of these compounds mainly occurs after longer storage period.

To further evaluate the effects of chitosan on wine aroma, a subset of samples was added of volatile phenols, to simulate a “Brett” contamination, and treated with KT and KTS for 7 days. Figure 2B reveals that powdered chitosan (KT) was able to decrease 4-EP and 4-EG by 10 and 40 µg/L respectively while higher performance was achieved with pre-dissolved chitosan (KTS), with reductions of 55 µg/L and 45 µg/L respectively. This different behavior may be linked to the higher specific surface induced by solubilization, promoting absorption capability of KTS toward volatile phenols.

Interestingly, both treatments showed a greater effect for 4-EG suggesting that the presence of polar methoxy group on 4-EG could stabilize the interactions with the polysaccharide leading to an increased absorption. These results were in contrast with those obtained by Milheiro and co-workers [10] where the total amount of volatile phenols was not significantly decreased in red wines but rather in the headspace concentration. This may be due to the fivefold higher dose of chitosan applied in our experiments revealing a dose dependent interaction with volatile phenols. Moreover, higher deacetylation degree used in our treatments seemed to increase chitosan performance, in accordance with results of Filipe-Ribeiro [9] because of the greater presence of amine (NH2) groups of glucosamine units to interact with volatile phenols.

However, it should be pointed out that 4-EP and 4-EG removal was limited, and remaining amounts in red wines were still substantially over the perception threshold (400 and 150 µg/L respectively [14]). According to this evidence chitosan could only be proposed as a remediating treatment for red wines with a low level of contamination of volatile phenols, potentially useful to bring their contents below the olfactory threshold. This result substantiates the cited findings [9,10,41] whereby chitosan was found to preferentially reduce the concentration of volatile phenols in the bottle headspace rather than in the wine itself.
were not able to detect any difference among samples, confirming that in highly contaminated wines, the removal capacity of chitosan towards ethyl phenols is not sufficient to reduce negative sensory impact imparted by them. Moreover, contrarily to what discussed for CIELab parameters showed in Table 3, panelists were not able to detect any difference in color intensity among samples.

Figure 2. (A) Quantification of volatile compounds grouped by chemical families and (B) quantification of 4-ethyl phenol (4-EP) and 4-ethyl guaiacol (4-EG) in wines after 7 days of treatment. In the same row, different letters indicate significant differences according to Tukey’s test ($p < 0.05$). $n = 3$. C: no addition; KT: 0.5 g/L of solid chitosan; KTS: 0.5 g/L of chitosan in solution.

3.5. Visual and Aroma Profile of Volatile Phenols-Spiked Wines after Treatments with Solid and Dissolved Chitosan

Figure 3 outlines the mean scores for each sensory attribute of samples spiked with ethyl phenols aiming to assess the sensory impact of Brett character. Since the level of addition of ethyl phenols greatly disturbed the taste analysis only an olfactory and visual description was carried out. Triangular test revealed that panelists were able to distinguish untreated samples among chitosan treated wines, while no differences were individuated between KT and KTS (data not shown). Judging by the results of Figure 3, very similar sensory profiles were obtained between the three samples, with only a slightly significative greater limpidity and herbaceous character in wines treated with dissolved chitosan (KTS). For this latter finding, the reduction in n-hexanol content after KTS addition (Table A1) may have played a role. In spite of the observed diminution of volatile phenols in chitosan-treated wines (Figure 2), the sensory attributes related to those volatiles (rubber and animal) were found not significantly different among samples, confirming that in highly contaminated wines, the removal capacity of chitosan towards ethyl phenols is not sufficient to reduce negative sensory impact imparted by them. Moreover, contrarily to what discussed for CIELab parameters showed in Table 3, panelists were not able to detect any difference in color intensity among samples.
Figure 3. Sensory profile of red wines spiked with 1500 µg/L ethyl phenol and 500 µg/L ethyl guaiacol after 7 days treatments with chitosan. CT: untreated; KT: solid chitosan; KTS: dissolved chitosan. * Indicates significant differences according to Tukey’s test (p < 0.05). n = 22.

4. Conclusions

The overall results showed that post fermentation fining of red wines with chitosan up to 500 mg/L could marginally influence the final color of the product mainly by reducing the co-pigmentation via absorption of the cofactors responsible of this phenomenon, namely hydroxycinnamic acids, flavonols and ellagic acid. At the doses adopted, titratable acidity of KT wines was up to 0.28 g/L lower than control samples, while KTS tended to affect this parameter to a lesser extent. The addition of chitosan did not appear to negatively impact the volatile components, also contributing to slightly decrease the concentration of 4-EP and 4-EG. Little differences were recorded in relation to the mode of addition and based on these results it can be concluded that, when used in red wines to reduce either contaminants or microbial spoilage, chitosan does not substantially impair the overall physical-chemical features and quality of wines. Further experiments are being developed by our group in order to assess its effectiveness on ageing, color stabilization and astringency of red wines.

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Appendix A

Table A1. Concentration of volatile compounds (µg/L) in red wines after 7 days of treatment. In the same row, different letters indicate significant differences according to Tukey’s test (p < 0.05). n = 3. C: untreated, KT: solid chitosan, KTS: dissolved chitosan. * Wines were added of volatile phenols.

| Alcohols              | C          | KT         | KTS        |
|-----------------------|------------|------------|------------|
| n-hexanol             | 833.58     | ab         | 795.30     |
| 3-penten-2-ol         | 808.86     | a          | 878.29     |
| 2-methyl-1-penten-3-ol| 30.18      | a          | 31.95      |
| 1-pentanol            | 42.06      | a          | 41.14      |
| 3-methyl-1-penten-3-ol| 24.98      | a          | 23.96      |
| 2-hexanol             | 282.74     | a          | 259.25     |
| 4-methyl-1-pentanol   | 18.42      | a          | 21.36      |
| 3-methyl-1-pentanol   | 42.54      | a          | 49.48      |
| n-hexanol             | 833.58     | ab         | 795.30     |
Table A1. Cont.

| Substance                              | C     | KT    | KTS   |
|----------------------------------------|-------|-------|-------|
| diacetone alcohol                      | 72.65 | a     | 102.87| a     |
| 3-ethoxy-1-propanol                    | 138.91| a     | 130.63| a     |
| 3-hexen-1-ol                          | 26.79 | a     | 27.23 | a     |
| 2-hexen-1-ol                          | 8.75  | a     | 11.63 | a     |
| butane-2,3-diol                        | 6027.26| a   | 5763.97| a | 5600.64| a |
| 1-octanol                              | 29.58 | a     | 25.14 | a     |
| 1-methoxy-2-butanol                    | 63.84 | a     | 59.45 | a     |
| 3-(methylthio)-1-propanol              | 555.91| a     | 541.36| a | 484.97| a |
| 3-(ethylthio)-1-propanol               | 30.09 | a     | 21.71 | a     |
| 2,7-dimethyl-4,5-Octanediol            | 8.75  | a     | 130.63| a | 67.58 | a |
| benzyl Alcohol                         | 6077.51| a   | 718.50 | a | 656.56| a |
| benzene ethanol                        | 41,839.71| a | 43,546.65| a | 41,945.85| a |
| 2,2-dimethyl-3-octanol                 | 44.87 | b     | 129.14| a | 83.82| ab |
| 4-ethyl-2-methoxyphenol*               | 704.68| a     | 663.59| b | 659.22| b |
| 4-methyl-phenol                        | 19.45 | a     | 15.91 | a     |
| 4-ethylphenol*                         | 1749.32 | a | 1738.24| b | 1696.44| c |
| Sum alcohol                            | 54,960.91| a | 56,563.91| a | 54,382.46| a |
| **Esters**                             |       |       |       |       |
| isoamyl acetate                        | 445.74| a     | 389.41| a | 344.64| b |
| ethyl caproate                         | 85.14 | b     | 102.31| a     |
| ethyl lactate                          | 5867.17| a   | 5905.26| a | 5400.47| a |
| ethyl octanoate                        | 13.39 | a     | 11.82 | a     |
| ethyl 3-hydroxy butanoate              | 221.71| a     | 232.53| a | 222.26| a |
| linalyl acetate                        | 40.22 | a     | 54.92 | a     |
| diethyl butanedioate                   | 4331.40| a   | 4243.50| a | 4033.41| a |
| 1,3-Propanediol, diacetate             | 444.05| a     | 430.85| a | 411.71| a |
| methyl 4-hydroxybutanate               | 51.09 | a     | 29.07 | a     |
| ethyl 1,4-hydroxybutanoate             | 3575.26| a   | 3805.39| a | 3594.69| a |
| β-phenylethyl acetate                  | 92.83 | a     | 111.59| a | 134.01| a |
| pentanoic acid, 2-methyl-pentyl ester   | 117.49| ab    | 141.38| a | 108.60| b |
| diethyl 2-hydroxypentanediode          | 372.02| ab    | 443.15| a | 344.12| b |
| glycine, N-acetyl, ethyl ester         | 0.00  | b     | 20.91 | a     |
| methyl hexadecanoate                   | 152.22| a     | 122.82| a | 150.46| a |
| ethyl 2-hydroxy-3-phenylpropionate     | 526.35| a     | 583.11| a | 527.07| a |
| methyl hydrogen succinate              | 91.85 | a     | 85.58 | a     |
| ethyl cinnamate                        | 626.91| a     | 416.33| b | 350.10| b |
| ethyl vanillate                        | 221.43| a     | 234.30| a | 211.84| a |
| Sum esters                             | 17,984.41| a | 17,958.84| a | 16,737.05| a |
| **Acids**                              |       |       |       |       |
| butanoic acid                          | 197.39| a     | 105.75| b | 93.41| b |
| isobutyric acid                        | 678.60| a     | 656.93| a | 629.77| a |
| pentanoic acid                         | 1120.94| a   | 1044.36| a | 1014.48| a |
| hexanoic acid                          | 874.99| a     | 900.37| a | 854.28| a |
| octanoic acid                          | 613.80| a     | 609.98| a | 588.10| a |
| decanoic acid                          | 73.46  | a     | 108.89| a | 91.08| a |
| benzoic acid                           | 35.80 | a     | 49.63 | a     |
| hexadecanoic acid                      | 374.62| a     | 362.08| a | 300.44| a |
| octadecanoic acid                      | 308.40| a     | 339.53| a | 227.47| a |
| Sum acids                              | 3598.49| a   | 3520.59| a | 3205.60| a |
| **Others**                             |       |       |       |       |
| 2-hydroxy-3-pentanone                  | 24.50 | a     | 25.72 | a | 29.06| a |
| Furfural                               | 10.04 | b     | 28.77 | a | 23.55| a |
| cis-5-hydroxy-2-methyl-1,3-dioxane     | 47.47 | a     | 43.75 | a | 44.12| a |
| benzaldehyde                           | 46.22 | a     | 52.57 | a | 43.08| a |
| linalyl acetate                        | 40.22 | a     | 54.92 | a | 29.20| b |
| β-citronellol                          | 23.72 | a     | 18.61| ab | 12.04| b |
| butyrolactone                          | 417.15| a     | 457.09| a | 397.76| a |
| 4-ethoxybutanolide                     | 127.88| a     | 111.08| a | 99.84| b |
| 2,6-pyran-2,6(3H)-dione                | 5.48  | a     | 9.39 | a     |
| pantolactone                           | 65.54 | a     | 70.38 | a | 66.49| a |
| 5-oxotetrahydrofuran-2-carboxylic acid, ethyl ester | 242.39| a | 367.09| a | 347.28| a |
| dihydro-5-(1-hydroxyethyl)-2(3H)-furanone | 374.57| b | 493.04| a | 405.37| ab |
| thiophene, 2,3-dihydro-                 | 413.42| a     | 375.67| a | 76.58| b |
| 2-furancarboxylic acid                 | 66.93 | a     | 73.59 | a | 62.23| a |
| vanillin                               | 136.62| a     | 107.07| a | 94.64| a |
| acetamid, N-(2-phenoxyethyl)-           | 43.71 | a     | 46.02 | a | 44.72| a |
| acetovinilnone                         | 90.06 | a     | 92.10 | a | 99.67| a |

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