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Influence of grape rot on the contents of sulfur binding compounds in wine after automated optical grape sorting

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Summary

In the last years, climate change has played an important role in some wine growing regions because of the increasing hazard of different kinds of bunch rot. *Botrytis cinerea* is the most important kind of rot on grapes. Beside sensory effects, this rot can influence the content of yeast nutrients, e.g. thiamine, in the must and thus affect the alcoholic fermentation. To get insight into the influence of *Botrytis cinerea* on the content of sulfur binding compounds formed during the fermentation process in wine, tons of grapes from the Mosel valley were sorted by an automated optical grape sorter, an innovative possibility of grape sorting, in 2011. Wine samples before sulfurisation of the four sorting fractions, namely control (unsorted berries), free-run (juice from opened berries), positive (healthy, intact berries) and negative (rotten berries) were analysed for the sulfur-binding compounds acetaldehyde, pyruvic acid, 2-oxoglutaric acid and for bound sulfur dioxide. The results show that acetaldehyde concentrations were not affected by rot, while pyruvic acid and 2-oxoglutaric acid levels were significantly higher in the negative fractions and lower in the positive fractions. Accordingly, bound sulfur levels were significantly higher in wines from the negative fraction. In conclusion, it could be shown that fractionation of the berries can efficiently help to reduce sulfur binding compounds in wine and thus reduce the addition of sulfur dioxide.

K e y w o r d s : acetaldehyde, pyruvic acid, 2-oxoglutaric acid, bound SO_2, grape sorting.

Introduction

The principal properties of sulfur dioxide (SO_2) in wine are antiseptic and antioxidant effects, inhibiting enzyme activity (e.g. tyrosinase and laccase) and protecting wine aromas by binding of fermentation by-products (Ribéreau-Gayon *et al.* 2006a). Total SO_2 is composed of bound and free SO_2, with the free form being the active one (Jakob *et al.* 1997). SO_2 content in grape juice and wine is regulated by law in the EU. According to the Commission Regulation (EC) No. 606/2009, the content of SO_2 is limited to 200 mg·L^{-1} in dry white wines with less than 5 g·L^{-1} residual sugar and to 250 mg·L^{-1} in white wines with more than 5 g·L^{-1} residual sugar. Wines with higher concentrations of sulfur dioxide are no longer marketable in the EU and consequently lead to economic loss. Therefore, it is important to minimize the content of sulfur binding compounds and so the addition of sulfur dioxide.

S u l f u r b i n d i n g c o m p o u n d s : Acetaldehyde, pyruvic acid and 2-oxoglutaric acid represent the most important sulfur binding compounds in must and wine (Fig. 1). They are considered as primary fermentation by-products of the alcoholic fermentation by yeasts (Jakob *et al.* 1997, Liu and Pilone 2000). The formation of these metabolites depends on the yeast species, the fermentation process and the constitution of the must, especially regarding the thiamine and the sugar contents (Barbe *et al.* 2000, Dittrich and Grossmann 2005).

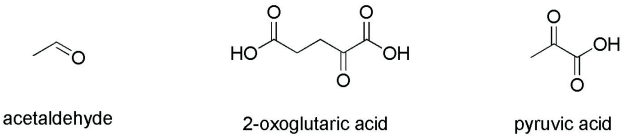

Fig. 1: Structures of sulfur binding compounds.

For the fermentation of glucose to ethanol by the yeast, thiamine as a constituent of the co-enzyme thiamine pyrophosphate is essential (Jakob *et al.* 1997). Bunch rot can influence this process because it metabolises the thiamine of the berries (Dittrich *et al.* 1974). Accordingly, the thiamine amount in must is reduced and the sulfur binding compounds cannot be further converted to ethanol. Higher concentrations of sulfur binding compounds require higher concentrations of sulfur dioxide for the preservation of the wine. As a consequence of higher values of bound SO_2, critical/illegal values of total SO_2 may be reached.

Botrytis cinerea is one of the principal pathogenic fungi of grapevines. Depending on the climatic conditions there are different states of this fungus, noble rot and gray mold being the most familiar ones. The chemical composition of grapes is greatly modified in the course of a *Botrytis* infection due to various enzymatic and metabolic processes. This fungus creates conditions favourable for the growth of other spoilage organisms (Dittrich and Grossmann 2005, Ribéreau-Gayon *et al.* 2006a).

At unfavourable meteorological conditions, *Botrytis cinerea* and acetic acid bacteria already cause an increased amount of sulfur binding compounds on the grapes (Dittrich and Grossmann 2005). In consequence, the binding of SO_2 can be twice as high in wines from botrytized berries as in wines from healthy berries (Dittrich et al. 1974).

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High temperatures and pH-values also favour the production of these compounds (Ribéreau-Gayon et al. 2006a).

Acetaldehyde represents the strongest binding partner since 1 mg binds 1.45 mg SO_2. In comparison 1 mg pyruvic acid binds 0.5 mg SO_2 and 1 mg 2-oxoglutaric acid binds 0.2 mg SO_2 (Dittrich and Grossmann 2005).

Higher amounts of acetaldehyde require higher amounts of sulfur dioxide because the binding of SO_2 leads to a decreasing amount of 'free SO_2' and so to weaker antimicrobial and antioxidative effects in wine (Jakob *et al.* 1997, Liu and Pilone 2000). The accumulation of acetaldehyde is not influenced by the available thiamine during the alcoholic fermentation as is the case with pyruvic acid and 2-oxoglutaric acid (Ribéreau-Gayon et al. 2006a).

In wine, acetaldehyde concentrations can vary between 6 and 170 mg·L^{-1} (Jakob *et al.* 1997). Acetaldehyde represents one of the most important sensorial carbonyl compounds formed during alcoholic fermentation. It constitutes more than 90 % of the total aldehyde content in wine (Nykanen 1986). At low levels it gives the wine a pleasant fruity aroma of apricot, banana and peach whereas, at higher concentrations, it leads to a green, grassy and apple-like off-flavour (Miyake and Shibamoto 1993, Ott *et al.* 1998, in Liu and Pilone 2000). Therefore, it is important to adjust the wines with sufficient 'free SO_2' to avoid an undesired sensorial influence of this compound.

Pyruvic acid is also formed during alcoholic fermentation as a precursor of acetaldehyde, the decarboxylation of pyruvic acid leading to acetaldehyde (Jakob *et al.* 1997, Dittrich and Grossmann, 2005). The most important factors affecting the content of pyruvic acid are the pH-value, grape variety, the content of thiamine and the yeast species (Rankine 1967, Rankine and Pocock 1969, Weeks 1969, Graham 1979, Barbe *et al.* 2000). In the presence of thiamine, the concentration of pyruvic acid and also the binding of SO_2 decrease. Thiamine ensures the decarboxylation of pyruvic acid to acetaldehyde. Thus, a lack of thiamine leads to an accumulation of keto acids in wine. Thiamine also represents an important nutrient for *Botrytis cinerea* so that an infection of the berries with this fungus results in a strong reduction of thiamine (Dittrich *et al.* 1974, Jakob *et al.* 1997, Ribéreau-Gayon *et al.* 2006a). In healthy musts, thiamine contents of 318 µg·L^{-1} were found whereas in mouldy musts only 35 µg·L^{-1} were determined (Dittrich and Grossmann 2005). According to the Community Regulation (EC) No. 606/2009 Annex I A, the addition of 0.6 mg·L^{-1} of thiamine to must is allowed. This addition is notably important to musts/wines from botrytized grapes (Jakob *et al.* 1997, Dittrich and Grossmann 2005). In German wines, pyruvic acid concentrations of up to 300 mg·L^{-1} were determined (Jakob *et al.* 1997). Sensorily it is described as being sour and influencing the mouth-feel and the body of the wine (Graham 1979).

2-oxoglutaric acid is formed in the course of the citric acid cycle of the yeast during its proliferation phase when it is used in various biosynthetic pathways (Jakob *et al.* 1997). This compound is also formed in higher concentrations in musts from botrytized berries (Dittrich *et al.* 1974, Dittrich and Grossmann 2005). In German wines, 2-oxoglutaric acid concentrations were reported to vary between 7 and 150 mg·L^{-1} (Jakob *et al.* 1997). The aim of this study was to find out to which extent rotten berries contribute to the formation of sulfur binding compounds during alcoholic fermentation. Therefore, automated optical grape sorting was used to separate healthy from rotten berries to determine this influence.

Sorting process: Grape sorting was performed with the automated optical grape-sorter Optyx 3375 (Key Technology, Walla Walla, Washington, USA) with an integrated high-speed RGB (red, green, blue) camera system (up to 4000 pictures per second) and an infrared laser (765 nm) for the detection of damaged berries and particles that reduce the quality to ensure and improve the wine quality (Fig. 2). To get representative results, amounts of grapes as obtained in actual practice from a whole vineyard, approximately two tons of grapes, were sorted and processed, thus resulting in authentic conditions. Prior to sorting, the machine has to be trained with healthy and rotten berries. A computer compares the data from the camera and the laser detection with the given data and sorts the grapes by blowing out "bad" (e.g. rotten) berries by air pressure in flight. Each sorting experiment was performed with the optimal calibration depending on the grape variety and type of rot.

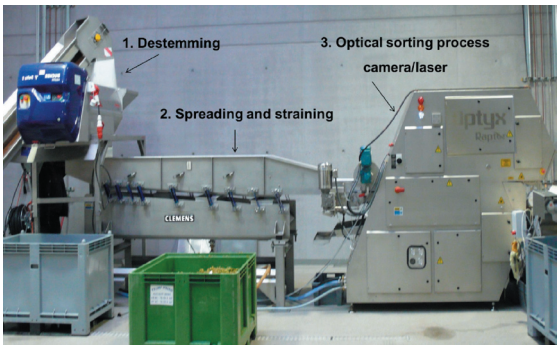

Fig. 2: Automated optical grape sorter at the DLR Mosel.

Different steps have to be performed before the optical scanning of the grapes (Fig. 2). The whole bunches are first destemmed (1) because the berries have to be singularized for detection and sorting. As further preparation steps, there is a spreading and a pressureless pre-straining of the berries on a vibration table where the free-run can drain off through the perforated plate of the table (2). White grape varieties, especially 'Riesling', can be sorted with an accuracy level of 98 % on average by our system, *i.e.* 98 % of rotten grapes from the grape crop can be separated, assessed by visual rating. The resulting fractions of such a sorting experiment are first the control 'fraction' (unsorted berries), a positive fraction (healthy and intact berries), a negative fraction (sorted and rotten berries) and a free-run (juice from opened berries, if existing).

Material and Methods

Samples: In 2011, different white grape varieties from different vineyards of the Mosel wine region were sorted and determined: 'Riesling' (Rsl), 'Müller-Thurgau'

(MTh) and 'Pinot gris' (PGr). The experimental grapes were harvested by hand or machine from 21st of September to 18th of October in 2011. The experiments are entitled with E1 to E8. The numbers of these individual experiments refer to the ascending order of the different harvest dates. During the processing of the grapes from the vintage up to the sorting process, mechanical forces affect the berries, i. e. mechanical or hand-picking harvest. The skins of botrytized berries, e.g., are unstable and can split easily. So, the juice in the form of the free-run can leak from the berries.

Climate: In fall of 2011, climate was warm and moist during the day with some tropical nights (over 20 °C). As primary infection *Botrytis cinerea* occurred, but secondary and tertiary infections like sour rot also occurred under these climatic conditions. Despite these subsequent infections, the amount of rot varied between 5 and 24 % (on average 13 %) in the control fractions (unsorted).

Sorting: The grapes were sorted by automated optical grape sorting as described above.

Vinification: Must and wine preparations were performed in a standardized manner without any finings. Sorting fractions were vinificated twice in 50 L stainless steel kegs per vinification. For fermentation, a white wine yeast of the species *Saccharomyces cerevisiae* was used. The fermentation process took 20 d on average at temperatures of 15 °C at the first half of this time and of 18 °C at the end. There was a sufficient nitrogen supply; thiamine was not added. Wine samples were taken 7 d after completed alcoholic fermentation, before sulfurisation.

Chemicals: The enzyme kits acetaldehyde and pyruvic acid were purchased from Megazyme International (Co. Wicklow, Ireland). Sodium hydroxide and ammonium sulphate were purchased from Merck (Darmstadt, Germany). Alpha-ketoglutaric acid disodium salt dihydrate and potassium dihydrogen phosphate were purchased from AppliChem GmbH (Darmstadt, Germany), ammonium dihydrogen phosphate, disodium hydrogen phosphate dodecahydrate and sodium hydrogen carbonate were purchased from Merck (Darmstadt, Germany), EDTA disodium salt dihydrate was purchased from Prolabo (Haasrode, Belgium), glutamate dehydrogenase (3000 U; from beef liver) and NADH disodium salt (100 % grade I) were purchased from Roche Diagnostics (Mannheim, Germany). All solutions and dilutions were prepared using demineralised water.

Methods: Acetaldehyde and pyruvic acid were determined enzymatically. Sample preparations were performed according to the assay procedures for wine, respectively (Megazyme International, Co. Wicklow, Ireland). Measurements of 2-oxoglutaric acid were performed according to the modified enzymatic method of Bergmayer and Bernt (1974). All samples were filtered through 0.45 µm cellulose-acetate-membranes (VWR; Darmstadt, Deutschland). Enzymatic measurements of the samples were performed in triplicate at 340 nm against water as a reference using a photometer Cary 60 UV-VIS (Agilent Technologies, Mulgrave, Victoria, Australia). The concentrations of acetaldehyde, pyruvic acid and 2-oxoglutaric acid were calculated as described in the assay procedures.

Bound sulfur dioxide measurements were carried out according to the Method OIV-MA-AS323-04B: R2009 (OIV, 2012).

Statistics: Statistics were performed using the two-way ANOVA with Tukey's post-hoc test with a significance level of $p \leq 0.05$. (Software: XLStatVersion 2013.4.04, Addinsoft, New York, USA). Data from wine samples of each experiment were statistically tested separately and marked with a, b, c and d.

Results

The four fractions of each sorting experiment (E1-E8) were abbreviated as follows: con = control, free = free-run, pos = positive fraction, neg = negative fraction. Each bar in the charts of the sulfur binding compounds shows the mean value of six replications of each fraction as result of the two vinifications, each analysed in triplicate, respectively. Different fermentation processes of both vinifications could result in the formation of different concentrations of sulfur binding compounds resulting in higher standard deviations.

Acetaldehyde concentrations in wines varied between 7.7 and 36.7 $mg \cdot L^{-1}$. There was no significant difference between the sorting fractions of one experiment. The maximum range within one experiment, *i.e.* in E7, was only 12.2 $mg \cdot L^{-1}$ between the negative and free-run fraction (Fig. 3).

Overall 2-oxoglutaric acid concentrations varied between 10 and 119 $mg \cdot L^{-1}$. There were only minor, but mainly significant (95 %) differences between the fractions of the experiments of E1, E5, E7 and E8. The negative fractions of E2, E3, E4 and E6 showed high concentrations of 2-oxoglutaric acid with clear significant differences (95 %) compared to the the control and the positive fractions (Fig. 4).

Pyruvic acid concentrations varied between 10 and 165 $mg \cdot L^{-1}$. Significant differences (95 %) could be observed between the fractions, with the highest pyruvic acid contents in the negative and the free-run fractions of the experiments E2, E3 and E6 (Fig. 5).

The bound SO_2 concentrations were highest in nearly all negative fractions, with the exception of E1 and E4. They varied altogether between 69 and 158 $mg \cdot L^{-1}$. The free-run fractions of the experiments E1, E2, E4, E6 and E8 showed second highest concentrations between 54 and 132 $mg \cdot L^{-1}$ bound SO_2. The lowest concentrations of bound SO_2 were mainly determined in the positive fractions with 49 to 80 $mg \cdot L^{-1}$ (Fig. 6).

Discussion

The results show that the degree of rottenness did not significantly influence the content of acetaldehyde. This is contrary to the conclusion of Dittrich *et al.* (1974) who determined 58 % higher acetaldehyde concentrations on average in wines from rotten grapes compared to wines from healthy ones. However, in individual cases, they were

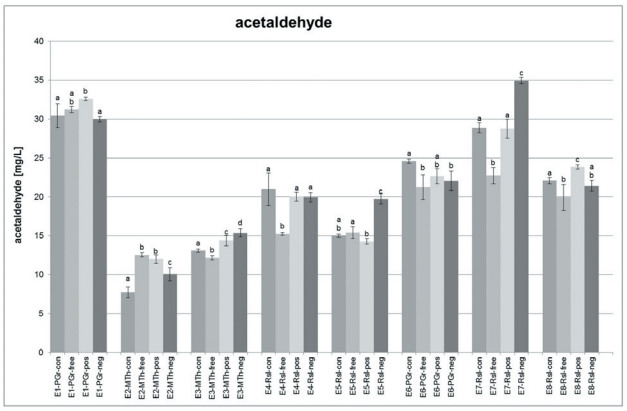

Fig. 3: Acetaldehyde concentrations in wine before sulfurisation in 2011 (n = 6; E1-E8: sorting experiments; con = control; free = free-run; pos = positive fraction; neg = negative fraction; PGr = 'Pinot Gris'; MTh = 'Müller-Thurgau'; Rsl = 'Riesling'; mv = mean value; s = standard deviation; statistics: wine samples of the four fractions from each sorting experiment were statistically tested separately and marked with a, b, c, d, $p \leq 0.05$).

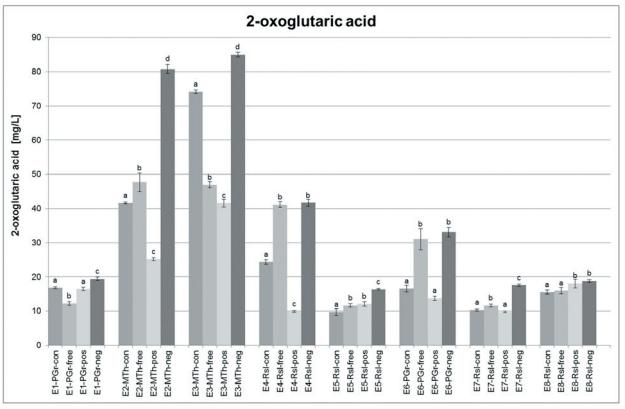

Fig. 4: 2-oxoglutaric acid concentrations in wine before sulfurisation in 2011 (n = 6; E1-E8: sorting experiments; con = control; free = free-run; pos = positive fraction; neg = negative fraction; PGr = 'Pinot Gris'; MTh = 'Müller-Thurgau'; Rsl = 'Riesling'; mv = mean value; s = standard deviation; statistics: wine samples of the four fractions from each sorting experiment were statistically tested separately and marked with a, b, c, d, $p \leq 0.05$).

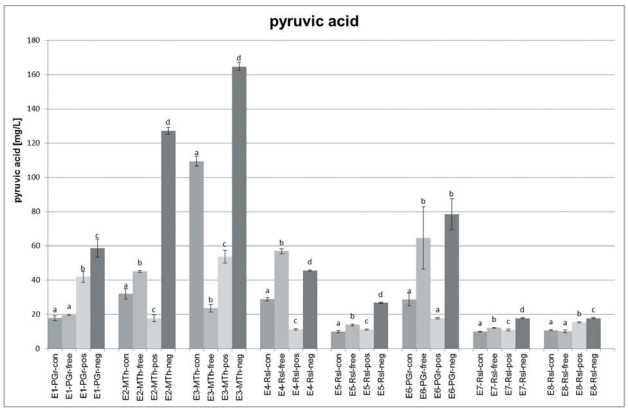

Fig. 5: Pyruvic acid concentrations in wine before sulfurisation in 2011 (n = 6; E1-E8: sorting experiments in 2011; con = control; free = free-run; pos = positive fraction; neg = negative fraction; PGr = 'Pinot Gris'; MTh = 'Müller-Thurgau'; Rsl = 'Riesling'; mv = mean value; s = standard deviation; statistics: wine samples of the four fractions from each sorting experiment were statistically tested separately and marked with a, b, c, d, $p \leq 0.05$).

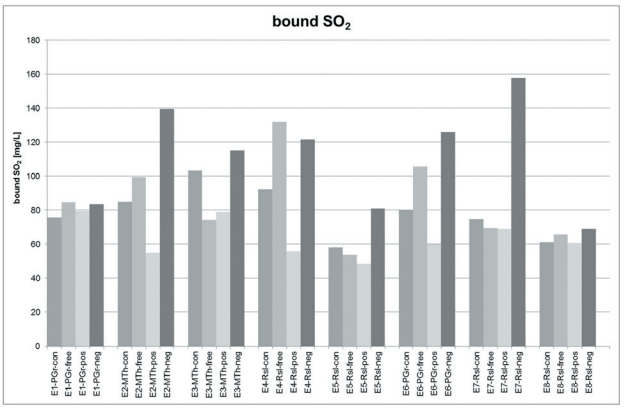

Fig. 6: Bound SO_2 in wine in 2011 (n =1; E1-E8: sorting experiments in 2011; con = control; free = free-run; pos = positive fraction; neg = negative fraction; PGr = 'Pinot Gris'; MTh = 'Müller-Thurgau'; Rsl = 'Riesling'; mv = mean value; s = standard deviation).

also not always able to detect differences between wines from healthy and rotten grapes.

In wine, acetaldehyde concentrations can vary between 6 and 170 mg·L^{-1} (JAKOB *et al.* 1997). In contrast, acetaldehyde concentrations of the sorting fractions varied in a clearly smaller range. The difference of these findings could be explained by the use of different yeast species, different fermentation conditions, different constitutions of the musts and the time of sulfurisation (after fermentation or during fermentation to get wines with residual sugar) since these factors are described to influence the formation of acetaldehyde (DITTRICH and BARTH 1984, BARBE *et al.* 2000, DITTRICH and GROSSMANN 2005).

Compared to the acetaldehyde concentrations the 2-oxoglutaric acid concentrations varied over a wider range. The higher concentrations in the negative fractions correlate with results from literature. DITTRICH *et al.* (1974) determined 2.5-times higher concentrations of 2-oxoglutaric acid in wines from rotten berries with 119 mg·L^{-1} on average compared to wines from healthy grapes with 50 mg·L^{-1} on average. This confirms the up to 3-times higher concentrations of the negative fractions compared to the positive fractions of our own experiments E2, E3, E4 and E6. Here 2-oxoglutaric acid is probably present in higher concentrations in musts from botrytized berries as a consequence of the lack of thiamine (DITTRICH *et al.* 1974, DITTRICH and GROSSMANN 2005).

In German wines pyruvic acid was found in concentrations up to 300 mg·L^{-1} (JAKOB *et al.* 1997). In this study we determined maximum values at about half of the maximum concentration reported. The results clearly show the influence of rot infections on the pyruvic acid concentrations. The negative fractions contained 3-times higher pyruvic acid concentrations on average than the positive fractions. This finding is in accord with the results of DITTRICH *et al.* (1974). As in the case of 2-oxoglutaric acid this can also be explained by a lack of thiamine. The *Botrytis* fungus uses thiamine as a nutrient which is thus not available as

coenzyme for the pyruvic acid decarboxylase. As a consequence, pyruvic acid cannot be decarboxylated to acetaldehyde and therefore accumulates (Dittrich *et al.* 1974, Jakob *et al.* 1997). In this study thiamine was not added to the sorting fractions during fermentation showing the influence of rot on this compound.

The high contents of bound SO_2 with 112 mg·L^{-1} on average in the negative fractions and the low contents with 63 mg·L^{-1} on average in the positive fractions also correlate with the findings of Dittrich *et al.* (1974), who found similar concentrations in musts from rotten and healthy berries. The binding of SO_2 of wines from botrytized grape material can be 2.5-times higher than of wines from healthy material (Dittrich *et al.* 1974). Acetaldehyde, pyruvic acid and 2-oxoglutaric acid represent the most important sulfur binding compounds (Jakob *et al.* 1997, Dittrich and Grossmann 2005). In the present study SO_2 is probably mainly bound by pyruvic acid and 2-oxoglutaric acid since these compounds showed higher concentrations compared to acetaldehyde. Especially the experiments E2 and E3 showing the highest pyruvic acid and 2-oxoglutaric acid concentrations also had high concentrations of bound SO_2. Higher contents of keto acids in botrytized wines cause a higher demand for SO_2 (Dittrich and Grossmann 2005).

According to the results for bound SO_2 and the concentrations of pyruvic acid and 2-oxoglutaric acid, the reduction of rotten grapes by automated optical grape sorting can lead to a lower dosage of SO_2 to wine.

Conclusion

In this study, automated optical grape sorting was successful concerning rot, mainly *Botrytis cinerea*, as the major sorting factor, what could be shown analytically by different contents of sulfur binding compounds in the sorting fractions. This innovative method enables sorting of tons of grapes, thus resulting in authentic, practical conditions. In this large-scale design for the first time, musts and wines from unsorted berries could be directly compared to those from the sorting fractions with sound vs. rotten berries from the same batch. The sorting of rotten grapes mainly caused lower concentrations of pyruvic acid and 2-oxoglutaric acid in wines from healthy berries, also resulting in lower concentrations of bound SO_2 compared to the control (unsorted) and negative fractions (rotten berries). Thus, sorting can avoid conflicts concerning the legal regulations for SO_2. In conclusion, automated optical grape sorting represents an innovation in the wine industry and enables quality control and improvement of the wines.

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