Impact of a combined pulsed electric field (PEF) and enzymatic mash treatment on yield, fermentation behaviour and composition of white wine

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Abstract

The aim of this work was to study the combined application of a pulsed electric field (PEF) and an enzymatic treatment to white wine mash. The resulting impact of membrane permeabilisation by electroporation and pectin degradation by enzymes on fermentation behaviour and quality of white wine was assessed. The mash of two varieties, Traminer and Grüner Veltliner, was PEF treated (3 and 10 kJ/kg) using a continuous co-linear treatment chamber. Pectinases were added immediately afterwards and maceration was performed for 4 and 24 h. Various physico-chemical parameters were analysed at different stages of the production process and the impact of the combined treatment on volatile compounds such as esters and terpenes was analysed by gas chromatography–mass spectrometry and a sensory panel in the final white wine after bottling and storage. Regardless of the PEF treatment intensity, the variety and the maceration time, the release of juice was not significantly influenced. For Traminer, the fermentation time was reduced through the enhanced extraction of nitrogen from 322 to 359 mg/L due to PEF treatment (10 kJ/kg). The release of phenols which were localized in the pulp, were significantly more affected than phenols from the skin. Although the concentration of selective esters especially of the variety Traminer significantly increased, the sensory evaluation indicated no positive effect on the olfactory properties. Overall, the combination of PEF and enzyme pretreatment showed benefits in reducing the fermentation time and increasing the content of selective esters for Traminer.

Keywords Pulsed electric field · Enzymes · White wine production · Yield · Aroma profile

Introduction

The challenges of modern wine production are mostly related to economic aspects like juice yield and fermentation behaviour as well as quality parameters such as aroma profile and sensory attributes. Both aspects require the extraction of valuable ingredients from grape berries. Therefore, efficient cell permeabilisation by mechanical disruption and degradation of cell wall compounds like pectin is required [1, 2]. It is well known that the quality of red and white

wines can be improved by extending the time of skin contact (maceration time). Whereas, by red wine production, the extraction of anthocyanins, polyphenols and volatile compounds is reached through the fermentation process on the skin (2–20 days), producers of white wines usually use enzymes to increase the juice yield and accelerate extraction of quality related components in less time (4–48 h) [3]. The commercial maceration enzymes are used for two different reasons: pectinases, cellulases and hemicellulases degrade hydrocolloids, which improves the juice yield and glycosidases break the connection of sugar-bonded aromatic compounds, thus increasing the extraction of volatile compounds of wines [4, 5]. Especially essential aroma precursors, such as 1-hexanol, nerol, geraniol and benzene derivative are located in the skin of the grape berries [6–8]. Hence, the processing of “aromatic” grape varieties, like Muscat, Gewürztraminer and Riesling, which also usually contain higher amounts of pectin, require the addition of pectinases. Moreover, for a sufficient separation of lees prior

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to the fermentation, the usage of pectinases are necessary and common. Wenzel, Dittrich [9] determined that in many cases, musts with higher turbidity tend to the formation of negative aroma components during the fermentation process, such as H₂S-like odours, which are related to rotten eggs.

In recent years, the usage of pulsed electric field (PEF) as an alternative for improving the juice recovery and extraction from various raw materials was frequently investigated and related benefits were described [10–12]. Thereby, unlike the application of enzymes, the enhanced extractability is related to the formation of pores in the cell membrane by electroporation. So far, the application of PEF pretreatment was mainly investigated for the production of red wines. Especially, aspects like fermentation behaviour, colour and the extraction of skin related components, such as polyphenols and anthocyanins, were studied [13–16]. The investigated parameters vary between the studies which make a direct comparison difficult. The applied field strength levels are generally lower than 1 kV/cm. Lebovka et al. [17] suggested a field strength of 400–600 V/cm for a sufficient treatment of grapes by PEF. However, the applied energy input ranges between 0.4 up to 120 kJ/kg [18, 19].

López-Giral et al. [20] found an increase in the content of specific polyphenols such as gallic acid (24%), caffeic acid (48%) and rutin (141%) after PEF pretreatment of red grapes of different varieties. A comparison of enzyme treatment and PEF treatment with regard to the impact on the extraction process was done by Donsì et al. [21] and revealed that for some of the investigated varieties, the enzyme treatment showed more pronounced effects. In contrast, Puértolas et al. [22] stated that the extraction of phenolic compounds from red grape mash after PEF treatment was more effective than the addition of enzymes.

Grimi et al. [23] reported that a PEF pretreatment ($E = 400$ V/cm $W \approx 15$ kJ/kg) of chardonnay grapes without the use of enzymes and with no additional time of skin contact increased the release of juice at constant pressure (1.0 bar) during pressing from 67 to 75%. Moreover, a 20% higher juice yield was achieved after PEF pretreatment ($E = 750$ V/cm $W \approx 20$ kJ/kg) of white grapes, whereby during the pressing process ($t = 45$ min) constant 5 bar were applied. Comuzzo et al. [24] describe a beneficial release of volatile compounds in white wine, such as terpenic and norisoprenoid glycosides by PEF ($E = 1.5$ kV/cm $W = 11$ and 22 kJ/kg). However, most of the analysed volatile compounds were below the olfactory threshold.

However, previous works only investigated the separate application of PEF and enzyme treatment for wine processing. Additionally, the effect of PEF on the extraction of polyphenols and volatile compounds was mainly described for red wines only. Only limited results about the impact of electric fields on the vinification process of white wines and quality related parameters, such as volatile compounds,

are available [24]. Therefore, the two different mechanisms, mechanical cell disruption by PEF and degradation of cell components (pectin) by enzymes were combined and their potential to improve the modern white wine processing was investigated in this work. Different PEF intensities (3 and 10 kJ/kg at 3 kV/cm) and maceration times (4 and 24 h) were compared regarding relevant parameters, like juice yield, fermentation behaviour, turbidity and general quality parameters including the content of phenols and volatile components, such as terpenes and esters.

Material and methods

Raw material

Grapes from *Vitis vinifera* L. var. Grüner Veltliner (GV) and Traminer (Tr) harvested in 2017 from Lower Austria were used. All samples from each variety were handpicked and selected on the same day. The grapes were harvested at the optimum ripening stage with regard to sugar content (°KMW) and acidity and stored for 12 h at 2 °C to achieve a temperature homogeneity in all batches. The grape material was randomized and separated in batches of similar size before further processing.

Pulsed electric field treatment and wine production

Prior to the application of pulsed electric field (PEF) treatment, single grape lots (≈ 30 kg) were destemmed and crushed. The mash (electrical conductivity 2 mS/cm) was pumped by an eccentric screw pump with a throughput of 440 kg/h through a co-linear treatment chamber (diameter 50 mm) consisting of three stainless steel electrodes (distance 43 mm), with the central electrode connected to high voltage and the outer electrodes connected to ground. The used PEF generator (ScandiNova Systems, Sweden) provides rectangular pulses with a pulse duration of 3 μ s. The PEF treatment parameters were selected in accordance to literature and preliminary tests in laboratory scale. Since a co-linear treatment chamber was used, the electric field strength is not homogeneously distributed. Hence, the average field strength was calculated based on Meneses et al. [25] and a value of 3 kV/cm was obtained and kept constant for all trials. The specific energy input (3 and 10 kJ/kg) was adjusted in dependence of the electrical current by changing the frequency. A total treatment time of 170 μ s and 502 μ s resulted for the lower and higher energy input level. Voltage and electric current were controlled using a digital oscilloscope (TBS 1102B-EDU, Tektronix, US). To prevent further mechanical disruption of the grapes during pumping, tubes with an inner diameter of minimum 50 mm were used. Immediately after PEF treatment, 3 g/hL pectolytic enzymes

(novoclar, Novozyme, Denmark) and 50 mg/L Sulphur dioxide were added to both PEF pretreated and untreated control mash and 4 and 24 h time of skin contact at 15 °C was realised. Pressing was realized in a small pneumatic press whereby the pressure regime was adapted to common industrial production conditions. Therefore, three consecutive steps were applied: (1) free juice release without additional pressure for 3 min, (2) moderate increase of pressure up to 1.5 bar for 3 min, (3) further pressure increase up to 2 bar for 3 min. After 12 h of spontaneous settling with the addition of 140 mg/L bentonite, clarification using a cellulose-based additive (Trubex neu, Erbslöh, Germany) was done. For standardized fermentation conditions, commercial yeasts for wine making (1118, Erbslöh, Germany) were added and the fermentation took place at a constant temperature of 18 °C. After completion of the fermentation, samples were centrifuged using a separator. For stabilization, 50 mg/L sulphur dioxide was added. Before bottling, wines were filtered, sulphur dioxide concentration was controlled and complemented to a concentration of 50 mg/L. Bottles were closed with screw caps and stored at 12 °C throughout the storage period until sampling after 3 and 10 months.

Juice yield

For the expression of juice yield in dependency of pressing pressure, the pretreated mash was weighted before pressing and the released juice was measured separately after each pressing step. According to Mannozi et al. [10], for the evaluation of the juice yield and the proportion (Y_x) of each pressing step, Eq. 1 was used, whereby m means the mass of juice and x indicates the respective pressing step.

$$Y_x = \frac{100}{\sum m} \times m_x \quad (1)$$

Turbidity of juice

Turbidity was measured immediately after pressing using a nephelometer (Turb 430, WTW, Weilheim, Germany) and expressed as nephelometric turbidity units (NTU), which describe the concentration of suspended particles in the grape juice [26]. Before the measurement, samples were diluted fourfold which was taken into account for the calculation.

Oenological parameters and fermentation behaviour

During the fermentation process, the fermentation behaviour was determined by measuring the density with a portable density meter (DMA 35, Anton Paar, Austria). Furthermore,

chemical parameters such as content of sugar (g/L), alcohol (%vol), total nitrogen (mg/L) and acids (g/L) were controlled periodically by FT-IR analyses, according to OIV/OENO Resolution 390/2010 using FOSS-WineScan (FT 120 Reference Manual, Foss, Hamburg, Germany).

Polyphenol content

Total polyphenol content (TPC) was determined after 10 months of storage using the FolinCiocalteu method according to Singleton et al. [27]. Therefore, 0.120 mL sample and 0.600 mL FolinCiocalteu reagent were mixed; after 2 min 0.960 mL Na_2CO_3 (7.5%) was added. After 5 min incubation at 50 °C, the mixture was cooled to room temperature and absorbance was measured with a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 760 nm. For calibration, a standard curve (0.01–0.10) with ferulic acid, was conducted. In addition, hydroxycinnamic acids were investigated by HPLC-method, according to Vrhovšek [28], with respect to the modification of Eder et al. [29] and Liszt et al. [30].

Aroma profile-volatile components

Esters

Selective esters were analysed 3 months after bottling using stable isotope dilution assay headspace solid-phase micro extraction gas chromatography-mass spectrometry (SIDA-HS-SPME-GC-MS). An adapted analytical method based on the procedure described by Philipp et al. [31] and Philipp et al. [32] was used. Therefore, a 7890A GC system (Agilent technologies, Paolo Alto, CA, USA) equipped with a DB-5 capillary column (60 m × 0.25 mm, 0.25 µm; stationary phase 5% dimethyl polysiloxane, 95% phenyl polysiloxane), a CombiPal autosampler (CTC analytics, Zwingen, Switzerland) and a 5975C MS detector (Agilent) were used. For the measurement, 1 mL sample, 2 mL Milli-Q water and 10 µL internal standard solution were mixed and transferred to a 20 mL head space vial containing 1 g NaCl (Zeller Hohenems, Austria). The internal standard solution includes (d5)-ethyl valerate, (d5)-ethyl hexanoate, (d5)-ethyl octanoate and (d5)-ethyl decanoate with an end concentration of 40 µg/L. For the headspace solid-phase microextraction (HS-SPME), a polyacrylate fibre (85 µm) from Sigma Aldrich, St. Louis, USA was used.

Terpenes

Selective free monoterpenes were analysed in the must before the fermentation started and after 3 months of storage by gas chromatography-mass spectrometry (GC/MS). The analytical procedure is based on the method described

by Michlmayr et al. [33]. For the measurement, the same equipment as previously described for esters was used. For analysis, 5 mL of sample and 10 µL internal standard 3,4-dimethylanisol, (Sigma Aldrich, St. Louis, USA), with an end concentration of 20 µg/L were mixed and combined in a headspace vial with 1.5 g NaCl (Zeller Hohenems, Austria). A 100 µm polydimethylsiloxane (PDMS) (Sigma-Aldrich, St. Louis, USA) was used for the headspace solid-phase microextraction (HS-SPME) as sample application system. The concentration of the sesquiterpene, rotundone of the variety Grüner Veltliner was separately analysed 3 months after bottling using a modified solid-phase microextraction gas chromatography–mass spectrometry method (SPE-SPME-GC-MS) as described by Nauer et al. [34].

Sensory

The sensory evaluation was conducted with a trained panel of 6 people and was performed 10 months after bottling. Wines were cooled to 9 °C and served at ambient temperature. During a preliminary sensory evaluation, the repetitions of each treatment were compared to verify possible variations. Repetitions of each treatment, which indicated no differences, were combined and used for the main sensory evaluation. Thereby, panellists described and evaluated each sample in two repetitions. To assess the sensory quality, international approved 20 point scheme and additionally descriptive free-choice analysis were implemented.

Statistics

Each pretreatment was repeated three times; chemical and physical analysis of each pretreatment were at least done in triplicates. Statistical tests were performed with Statgraphics Centurion XVIII (Statpoint Technologies, Virginia, USA). The plotted results are presented as mean value $\pm$ SD. The significance level for analyses of variance (ANOVA) was set to $p < 0.05$. LSD post hoc comparison testing was used to identify significant differences of means.

Results and discussion

Juice yield

The results in Fig. 1 describe the influence of PEF and enzymatic mash treatment on juice yield of Grüner Veltliner and Traminer in dependency of maceration time and over the course of applied pressing conditions. For both varieties, no relevant differences between the treated (PEF + enzymes) and untreated (only enzymes) mash, independent from the maceration time were detectable. The additional application of PEF pretreatment does not lead to higher overall juice

Fig. 1 Dependence of juice yield of Grüner Veltliner and Traminer related to different pressing conditions and PEF intensities obtained from pressing after 4 and 24 h maceration time with skin contact

yield and does also not show a difference in juice yield for the pressing stages operating at lower pressure. Hence, an adaptation of the pressure profile cannot be considered as a mean to convert the cell permeabilisation achieved by PEF into higher juice yields in this case. The extension of the maceration time was found to contribute to increase the juice yield. However, no effect of PEF was detected for the longer maceration times and the potential benefit of PEF in reducing maceration times could not be found. López et al. [13] studied the vinification of Tempranillo grapes and showed the possibility of reducing the maceration time by applying a PEF treatment. Also, Praporscic et al. [35] determined an increase of juice yield by PEF ($E = 750$ V/cm $W \approx 20$ kJ/kg) for white grapes from 49–54 to 76–78%, after pressing with constant 5 bar for 45 min. Jaeger et al. [36] describe an increased juice release for carrots (8–31%) and apples (0–11%) already at lower pressure. Different pressing systems, raw materials and mash structures were used and make direct comparison of results difficult. However, most of the studies detecting an increased juice release by

the application of electric fields used raw materials without any kind of common pretreatments, such as the addition of enzymes, as control for comparison. Taking into account that enzymatic mash treatment increases the juice release by the degradation of pectin and facilitates the extraction process, its application is crucial to modify the mash structure and viscosity as intended during conventional vinification. Furthermore, the beneficial effect of PEF in comparison to untreated mash related to extraction processes, was described as much more distinct immediately after the pretreatment, and decreases by extended time of skin contact. Whereby the differences can be related to the activity of native and added pectinases and osmotic effects during the fermentation process. Delsart et al. [16] detected that the application of PEF pretreatment with 4 kV/cm leads to an increase in polyphenols with only 11% remaining after alcoholic fermentation with skins, whereby the measured concentration in the same must (before fermentation) was 55% higher compared to untreated grapes. Although, a PEF treatment can contribute to the disintegration of the cell membrane and to the release of intracellular compounds, the impact of conventional processing steps such as the application of enzymes or the alcoholic fermentation with skins, remain relevant to generate desired product properties. Hence, PEF effects are superimposed by the other processing steps and benefits with regard to juice yield increase cannot be maintained.

Fermentation behaviour

For the wine industry, a rapid fermentation and complete conversion of sugar into alcohol are of main importance for reasons of product quality and process efficiency. The present investigations show that PEF pretreatment reduces the fermentation time to a small extent. In case of the variety Traminer, the duration of the fermentation could be reduced from 16 to 14 days or from 20 to 16 days depending on the maceration time of 4 or 24 h, respectively (Table 1). Thereby, the applied energy input during PEF treatment shows no significant effect. However, the fermentation of the variety Grüner Veltliner was not affected by the PEF

pretreatment. Independent from the time of skin contact and pretreatment intensity, the alcoholic fermentation lasted approximately 30 days.

The impact of PEF regarding the reduction of fermentation time could be related to the facilitated extraction of nitrogen. The recent investigations show that, especially for Traminer, beside the extended maceration time, also the application of PEF leads to a significantly higher release of total nitrogen (Fig. 2). The content increased by about 10%, whereby no significant differences between both energy inputs were detectable. Previous investigations showed that immediately after PEF pretreatment, the ammonium concentration and the amino acid content of Parellada and Garganega grape juice are not affected in comparison to untreated samples [24, 37]. However, different varieties were investigated and no maceration was applied.

For the alcoholic fermentation, the presence of assimilable nitrogen is of main importance and it is considered as a limiting nutrient for yeasts [38]. Particularly, lower nitrogen content during the fermentation leads to reduced yeast population and increased occurrence of stuck and slow fermentations [39]. Furthermore, the formation of negative volatile sulphur compounds with a characteristic odour of rotten eggs, putrefaction, onion and garlic, undesirable thiols and higher alcohols, is favoured, whereas desirable esters and long chain fatty acids decrease [40, 41].

Turbidity

The commonly used method for clarification before fermentation is spontaneous settling, whereby pectolytic enzymes are often added for the degradation of pectin and to decrease the processing time [42]. Effects of PEF and enzyme treatment on juice turbidity immediately after pressing measured

Table 1 Duration of fermentation (days) depending on pretreatment, variety and maceration time

Variety	Maceration time (h)	Control	Fermentation time (days)	
			3 kJ/kg	10 kJ/kg
Grüner Veltliner	4	31 ± 1	31 ± 0	30 ± 1
	24	31 ± 1	31 ± 1	30 ± 1
Traminer	4	16 ± 0	14 ± 1	15 ± 1
	24	20 ± 2	16 ± 1	16 ± 1

Fig. 2 Total nitrogen content of Grüner Veltliner and Traminer depending on pretreatment and maceration time. Means with different letters per variety are significantly different ($p < 0.05$)

in the must are shown in Fig. 3. Especially, findings for the variety Traminer indicate a significantly higher degree of turbidity (17%) after combined PEF and enzyme treatment in comparison to the control for which enzyme treatment only was applied. The increase was not affected by the time of skin contact. In contrast, no distinct effect of PEF was detectable for Grüner Veltliner independent of maceration time and energy input. Higher turbidity and negative effects on sedimentation were also detected after PEF treatment of Garganega grapes [24]. However, investigations from Praporscic et al. [35] showed a decrease of juice turbidity and the content of solid particles after the application of PEF for different white grape varieties. Additionally, Grimi et al. [23] do not note any effects of PEF on the turbidity of white grape juice independent from the applied pressing conditions. Singleton et al. [43] and Koenitz et al. [44] investigated the influence of higher turbidity degrees during fermentation on sensory properties of different white wines. Thereby, an expert panel of tasters described wines prepared from clarified juices as higher in quality and with a more intense aroma than wines fermented with higher amounts of suspended solids. The turbidity leads to increased astringency and bitterness, which was described by the panel as a harsh taste with additional off odours, especially hydrogen sulphide. Lower turbidity during the fermentation limits the release of fusel alcohols, which leads to a typical wine aroma quality [42]. Therefore it is of main importance for the production of high-quality wines to minimize the presence of lees during the fermentation. Due to the increased turbidity achieved by PEF, the clarification process needs to be more efficient which could be realized by adding higher enzyme concentrations and/or an additional clarification step such as separation or filtration systems, both come along with higher production costs and time exposure.

Fig. 3 Dependence of turbidity of Grüner Veltliner and Traminer regarding energy input and maceration time. Means with different letters are significantly different ($p < 0.05$)

Impact of PEF and enzyme pretreatment on hydroxycinnamic acids

In white wine, hydroxycinnamic acids are the main class of polyphenols, whereby caftaric acid, *p*-coumaric acid and fertaric acid are the most important ones. *Trans*-caftaric acid and *trans*-fertaric acid are easier to release during pressing due to their presence in the pulp, whereas *cis* and *trans p*-coumaric acids are localized in the skin and hence less extractable [45, 46]. In Table 2, the total polyphenol content (TPC) and the concentration of selected polyphenols especially hydroxycinnamates are shown for Grüner Veltliner and Traminer after 10 months of storage. Almost all analytes increased by the application of PEF are independent from the variety. However, due to PEF, the release of phenols which were localized in the pulp was significantly more affected than phenols from the skin. For Grüner Veltliner, *c*-coumaric acid (skin) was increased by application of PEF (10 kJ/kg) and enzymes of about 23% whereas *trans*-caftaric acid (pulp) showed an increase of 96%, in comparison to the sample pretreated by enzymes only.

The facilitated extraction of phenols from red grapes due to PEF is described as a major positive effect due to the resulting enhanced intensity of colour and mouth feeling [47]. The application of PEF on Merlot grapes resulted in an increased release of selective phenols and additionally improved the sensory attributes. Additionally, the enhanced release of total phenols was described in previous literature as a potential health benefit [3, 48].

However, in the case of white wine, increased polyphenol contents often lead to higher astringency, bitterness and accelerated ageing [8]. The present results showed that the negative effect of the applied pretreatment regarding the increased polyphenol content is significantly higher for both varieties after 24 h of maceration. Enhancement of the energy input from 3 to 10 kJ/kg only leads to an increase of approximately 10% of *t*-coumaric acid for Grüner Veltliner after 24 h whereas the extension of maceration time from 4 to 24 h (at 10 kJ/kg), resulted in an increase of 32%. In addition, an increase of the specific energy input did not lead to a higher release of polyphenols in general. As already described for red grape skins, the impact of the applied PEF intensity on the extraction of polyphenols, anthocyanins and colour intensity depended on the grape variety and the initial composition. Increasing the field strength from 2 to 10 kV/cm leads to a significant improvement for Mazuelo, whereas it did not show improvements for Garnacha and Graciano [19, 20]. Therefore, in case of implementation for industrial use, it is essential to define an appropriate combination of the PEF parameters and the time of maceration in dependency of the variety.

Table 2 Total polyphenol content (TPC) and overview of selective polyphenolic compounds (mg/L) of Grüner Veltliner and Traminer after 10 months storage period related to varied pretreatments and maceration times (mean value $\pm$ SD)

Variety	Maceration time (h)	Treatment	TPC	Caffeic acid	<i>o</i> -Coumaric acid cal. a	<i>p</i> -Coumaric acid cal. a	Catechin	Procyanidin B1	Ferulic acid cal. a	Epicatechin	Procyanidin B2	Ferulic acid
Grüner Veltliner	4	Control	306 $\pm$ 50.01a	34.04 $\pm$ 7.85a	17.61 $\pm$ 2.53a	6.46 $\pm$ 2.14d	23.85 $\pm$ 2.54a	2.35 $\pm$ 0.72a	4.47 $\pm$ 0.41a	1.04 $\pm$ 0.07a	2.8 $\pm$ 0.55a	3.51 $\pm$ 1a
		3 kJ/kg	364.67 $\pm$ 5.32ab	53 $\pm$ 1.46b	21.69 $\pm$ 0.67b	11.63 $\pm$ 0.38ab	29.35 $\pm$ 1.8b	2.72 $\pm$ 0.14a	5.21 $\pm$ 0.0bc	1.22 $\pm$ 0.04b	2.89 $\pm$ 0.02ab	3.98 $\pm$ 0.13a
	24	Control	378 $\pm$ 15.95b	66.82 $\pm$ 2.04c	21.69 $\pm$ 0.23b	14.8 $\pm$ 0.82a	26.64 $\pm$ 2.21ab	2.62 $\pm$ 0.18a	5.62 $\pm$ 0.04 cd	1.4 $\pm$ 0.03c	3.13 $\pm$ 0.1abc	3.89 $\pm$ 0.25a
		3 kJ/kg	403.33 $\pm$ 26.46b	44.26 $\pm$ 7.35ab	18.15 $\pm$ 0.92a	9.61 $\pm$ 1.85bd	37.53 $\pm$ 0.71c	4.51 $\pm$ 0.91b	4.87 $\pm$ 0.38ab	1.35 $\pm$ 0.01c	2.77 $\pm$ 0.33a	5.92 $\pm$ 0.8b
Traminer	4	Control	528 $\pm$ 51.54c	78.72 $\pm$ 9.58 cd	23.4 $\pm$ 0.85b	19.74 $\pm$ 1.79c	42.48 $\pm$ 1.96d	6.51 $\pm$ 0.7c	5.92 $\pm$ 0.18d	1.63 $\pm$ 0.03d	3.67 $\pm$ 0.29bc	8.04 $\pm$ 0.89c
		3 kJ/kg	536.67 $\pm$ 18.83c	87.69 $\pm$ 3.72d	24.12 $\pm$ 1.31b	21.84 $\pm$ 1.45c	42.76 $\pm$ 0.92d	6.72 $\pm$ 0.78c	6.03 $\pm$ 0.07d	1.66 $\pm$ 0.04d	3.49 $\pm$ 0.13c	7.9 $\pm$ 0.46c
	24	Control	281 $\pm$ 12.8a	7.27 $\pm$ 2.39a	14.98 $\pm$ 0.48a	1.79 $\pm$ 0.25a	15.81 $\pm$ 1.72a	4.19 $\pm$ 0.66x	2.83 $\pm$ 0.16ab	1.2 $\pm$ 0.05a	2.17 $\pm$ 0.31a	4.49 $\pm$ 1.24a
		3 kJ/kg	380 $\pm$ 14.53b	5.49 $\pm$ 0.25a	18.06 $\pm$ 0.74b	1.87 $\pm$ 0.16a	17.93 $\pm$ 1.58a	5.54 $\pm$ 0.31b	2.81 $\pm$ 0.02a	1.45 $\pm$ 0.03b	2.6 $\pm$ 0.28ab	5.73 $\pm$ 0.72a
	4	Control	386 $\pm$ 25.01b	6.22 $\pm$ 0.41b	17.93 $\pm$ 0.26b	1.98 $\pm$ 0.19a	18.81 $\pm$ 1.28a	5.36 $\pm$ 0.12b	2.97 $\pm$ 0.01abc	1.61 $\pm$ 0.16b	2.67 $\pm$ 0.08b	5.96 $\pm$ 0.68a
		3 kJ/kg	432 $\pm$ 14.53c	7.99 $\pm$ 0.19c	16.54 $\pm$ 0.66c	2.44 $\pm$ 0.13b	25.51 $\pm$ 0.18b	7.08 $\pm$ 0.23c	2.92 $\pm$ 0.07abc	1.55 $\pm$ 0.01b	3.18 $\pm$ 0.04c	9.7 $\pm$ 0.44b
	24	Control	466 $\pm$ 17.66c	10.52 $\pm$ 0.29d	18.58 $\pm$ 0.48b	3.35 $\pm$ 0.07c	28.47 $\pm$ 2.06bc	8.54 $\pm$ 0.7d	3 $\pm$ 0.06bc	2.12 $\pm$ 0.09c	3.8 $\pm$ 0.23d	11.27 $\pm$ 1.04bc
		3 kJ/kg	506.67 $\pm$ 24.61d	11.31 $\pm$ 0.24e	18.25 $\pm$ 0.6b	3.57 $\pm$ 0.17c	29.43 $\pm$ 1.03c	8.82 $\pm$ 0.51d	3.09 $\pm$ 0.04c	2.18 $\pm$ 0.04c	3.83 $\pm$ 0.17d	11.57 $\pm$ 0.54c

Means with different letters per variety are significantly different ($p < 0.05$)

Volatile compounds and sensory evaluation

The olfactory attributes of wine are related to more than 800 volatile compounds, whereby some are already present in the grapes, some are formed during the alcoholic fermentation and others are developed during ageing [49]. The different odoriferous compounds are located both in the skin and in the pulp, whereby their distribution depends on the variety [50]. The usage of enzymes during the maceration time results in significant higher contents of free terpenes and norisoprenoids as compared to the application of clarification enzymes, which is due to the additional glycosidase activity of maceration enzymes [51].

In general, the aroma composition of the variety Grüner Veltliner was not significantly affected by the combined application of PEF and enzymes. Almost all tested esters (Table 3) and terpenes (Table 4) indicated low levels and no differences due to the PEF treatments at different intensities were detectable. Only the esters, ethyl butyrate and acetic acid-2 and 3-methylbutyl ester were enhanced by the PEF treatment. In addition, the extension of the maceration time did not result in an increased concentration of esters. For the specific “peppery” character of the variety Grüner Veltliner, the sesquiterpene rotundone is responsible. Approximately 99% of the total rotundone of Grüner Veltliner is placed in the exocarp [52]. In light of this fact, extended skin contact is able to increase the release of this specific sesquiterpene. In the present study, the content of rotundone for the variety Grüner Veltliner was not positively affected neither by the applied PEF pretreatment nor by extending the maceration time from 4 to 24 h (Fig. 4). This can be explained by the comparably low amount of the sesquiterpene measured in the wine of only 11 ng/L, which is related to the used variety clone, climate and vintage. The concentrations of rotundone of Austrian Grüner Veltliner can range from 9.5 to 84.7 ng/L [34]. For the investigated conditions, no benefit from the application of PEF or extended maceration could be derived.

As already mentioned and described for polyphenols, the effect of pretreatments with electric fields on volatile compounds also depends on the variety. Garde-Cerdán et al. [15] described an enhanced amount of monoterpenoids, β -ionone, total esters and benzenoid compounds for Grenache, whereas the quantity of volatile components of Tempranillo and Graciano was not increased by PEF pretreatment. Differences depending on variety were also found in the present study, where the effects of a PEF pretreatment were significantly higher for Traminer compared to Grüner Veltliner. By the application of 10 kJ/kg and 24 h maceration time, the concentration of selective esters such as ethyl-*trans*-2-decanoate (+150%), ethyl dodecanoate (+110%) and ethyl decanoate (+87%) got significantly increased compared to the samples treated by enzymes only. For all selected terpenes, a significant increase for PEF treated grapes (3 kJ/kg) compared

Table 3 Concentration of selected esters ($\mu\text{g/L}$) of Grüner Veltliner and Traminer after 3 months of storage related to different pretreatment and maceration time (mean value $\pm$ SD)

Variety	Macera- tion time (h)	Treatment	Pentyl acetate	Acetic acid 2 methylpropyl ester	Acetic acid 2and 3-methylbutyl ester	Ethyl butyrate	Pentyl butyrate	Methyl octanoate	Methyl <i>trans</i> - geranoate	Butyl butanoate	Hexyl acetate
Grüner Veltliner	4	Control	n.d	191.78 $\pm$ 79.25a	857.78 $\pm$ 50.44b	130.23 $\pm$ 14.34a	1.65 $\pm$ 0.27b	2.48 $\pm$ 0.31a	n.d	30.87 $\pm$ 1.19a	181.77 $\pm$ 38.79ab
		3 kJ/kg	n.d	131.82 $\pm$ 19.27a	852.17 $\pm$ 16.27b	345.55 $\pm$ 91.7b	1.14 $\pm$ 0.02a	2.4 $\pm$ 0.57a	n.d	30.06 $\pm$ 2.54a	213.07 $\pm$ 47.09ab
		10 kJ/kg	n.d	190.56 $\pm$ 9.01a	1257.12 $\pm$ 83.35c	338.37 $\pm$ 40.51b	1.22 $\pm$ 0.06a	2.29 $\pm$ 0.18a	n.d	28.79 $\pm$ 2.37a	227.1 $\pm$ 10.48b
	24	Control	n.d	121.85 $\pm$ 20.42a	566.47 $\pm$ 116.69a	251 $\pm$ 32.78b	1.45 $\pm$ 0.34ab	3.12 $\pm$ 1.42a	n.d	30.97 $\pm$ 0.59a	148.31 $\pm$ 39.21a
		3 kJ/kg	n.d	127.99 $\pm$ 37.56a	812.44 $\pm$ 84.5b	275.94 $\pm$ 11.51b	1.15 $\pm$ 0.1a	2.99 $\pm$ 0.77a	n.d	27.21 $\pm$ 0.74a	172.99 $\pm$ 28.57ab
		10 kJ/kg	n.d	159.91 $\pm$ 25.64a	959.54 $\pm$ 5.14b	267.85 $\pm$ 26.46b	1.18 $\pm$ 0.14a	2.46 $\pm$ 0.76a	n.d	27.99 $\pm$ 1.98a	172.42 $\pm$ 34.32ab
Traminer	4	Control	15.5 $\pm$ 0.94b	263.51 $\pm$ 29a	3160.85 $\pm$ 41.4b	538.37 $\pm$ 25.52a	1.85 $\pm$ 0.1a	3.12 $\pm$ 0.07a	2.7 $\pm$ 0.75a	27.68 $\pm$ 0.13b	417.7 $\pm$ 26.99a
		3 kJ/kg	14.43 $\pm$ 2.97b	347.89 $\pm$ 8.16ab	3073.59 $\pm$ 25.27a	620.36 $\pm$ 95.78ab	2.27 $\pm$ 0.36a	2.87 $\pm$ 0.23a	3.08 $\pm$ 0.27ab	27.76 $\pm$ 0.11b	434.71 $\pm$ 34.11ab
		10 kJ/kg	11.06 $\pm$ 1.64ab	455.78 $\pm$ 66.42bc	3053.09 $\pm$ 35.67a	563.4 $\pm$ 60.32a	2.32 $\pm$ 0.53a	3.34 $\pm$ 0.14a	4.03 $\pm$ 0.11b	27.78 $\pm$ 0.12b	461.21 $\pm$ 10.2ab
	24	Control	23.21 $\pm$ 2.59c	464.31 $\pm$ 38.92c	3150.35 $\pm$ 37.51b	569.62 $\pm$ 47.73a	2.25 $\pm$ 0.09a	5.03 $\pm$ 0.82b	7.01 $\pm$ 0.13c	27.13 $\pm$ 0.4a	521.33 $\pm$ 93.14ab
		3 kJ/kg	13.58 $\pm$ 1.48ab	512.2 $\pm$ 52.84c	3483.59 $\pm$ 22.52c	733.06 $\pm$ 35.5b	2.18 $\pm$ 0.23a	4.64 $\pm$ 0.09b	6.63 $\pm$ 1.09c	27.87 $\pm$ 0.07b	496.05 $\pm$ 28.43ab
		10 kJ/kg	9.73 $\pm$ 2.16a	486.16 $\pm$ 86.13c	3482.28 $\pm$ 34.47c	644.14 $\pm$ 83.81ab	1.93 $\pm$ 0.2a	4.91 $\pm$ 0.43b	7.06 $\pm$ 0.23c	27.78 $\pm$ 0.03b	540.97 $\pm$ 70.69b
Variety	Macera- tion time (h)	Treat- ment	Ethyl hexanoate	Octyl acetate	Hexanoic acid 3-methylbutyl ester	Octanoic acid propyl ester	Methyl decanoate	Ethyl octanoate	Ethyl- <i>trans</i> - 2-decenoate	Ethyl dode- canoate	Ethyl decanoate
Grüner Velt- liner	4	Control	759.82 $\pm$ 104.16a	1.74 $\pm$ 0.53a	1.28 $\pm$ 0.21a	n.d	2.2 $\pm$ 0.32a	1674.37 $\pm$ 230.68a	4.41 $\pm$ 0.14a	65.71 $\pm$ 9.43a	1069.95 $\pm$ 86.39a
		3 kJ/kg	887.27 $\pm$ 190.73a	1.9 $\pm$ 0.3a	1.68 $\pm$ 0.37ab	n.d	2.01 $\pm$ 0.49a	1994.13 $\pm$ 511.22a	7.57 $\pm$ 1.87bc	84.11 $\pm$ 9.83bc	1315.82 $\pm$ 334.15ab
		10 kJ/kg	903.68 $\pm$ 54.64a	1.82 $\pm$ 0.12a	1.99 $\pm$ 0.09b	n.d	1.9 $\pm$ 0.14a	2277.51 $\pm$ 104.62a	12.19 $\pm$ 1.35d	90.75 $\pm$ 2.19c	1517.98 $\pm$ 50.03b
Traminer	24	Control	799.71 $\pm$ 158.8a	2.66 $\pm$ 0.98a	1.27 $\pm$ 0.2a	n.d	2.92 $\pm$ 1.28a	1774.93 $\pm$ 307.65a	6.33 $\pm$ 0.43ab	55.41 $\pm$ 8.85a	1081.05 $\pm$ 112.3a
		3 kJ/kg	805.9 $\pm$ 146.22a	1.8 $\pm$ 0.39a	1.27 $\pm$ 0.12a	n.d	2.64 $\pm$ 0.63a	1775.5 $\pm$ 331.68a	8.26 $\pm$ 1.3bc	88.96 $\pm$ 10.12bc	1221.33 $\pm$ 202.89ab
		10 kJ/kg	790.93 $\pm$ 119.01a	2.22 $\pm$ 0.17a	1.34 $\pm$ 0.07a	n.d	2.23 $\pm$ 0.6a	1780.61 $\pm$ 153.65a	9.06 $\pm$ 0.49c	72.71 $\pm$ 2.69ab	1186.19 $\pm$ 95.04ab
	4	Control	1091.85 $\pm$ 27.17a	4.35 $\pm$ 0.35ab	1.16 $\pm$ 0.04a	2.1 $\pm$ 0.13a	2.47 $\pm$ 0.08a	2268.78 $\pm$ 35.66a	6.81 $\pm$ 0.89a	70.99 $\pm$ 12.85a	1426.48 $\pm$ 190.44a
		3 kJ/kg	1234.13 $\pm$ 22.16ab	3.49 $\pm$ 0.26a	1.32 $\pm$ 0.15ab	2.75 $\pm$ 0.29b	2.29 $\pm$ 0.15a	2405.78 $\pm$ 206.02a	8.3 $\pm$ 0.64a	92.2 $\pm$ 4.47a	1730.6 $\pm$ 117.33a
		10 kJ/kg	1175.94 $\pm$ 28.28ab	4.23 $\pm$ 0.25ab	1.87 $\pm$ 0.1c	3.23 $\pm$ 0.19b	2.77 $\pm$ 0.17a	2462.8 $\pm$ 276.79a	12.42 $\pm$ 0.63b	128.51 $\pm$ 13.47b	2332.88 $\pm$ 281.24b
	24	Control	1288.5 $\pm$ 155.68b	3.9 $\pm$ 0.83ab	1.43 $\pm$ 0.1abc	2.87 $\pm$ 0.38b	4.09 $\pm$ 0.69b	2653.06 $\pm$ 306.28a	7.42 $\pm$ 0.78a	77.02 $\pm$ 17.22a	1710.6 $\pm$ 58.26a
		3 kJ/kg	1148.16 $\pm$ 36.03ab	4.18 $\pm$ 0.25ab	1.62 $\pm$ 0.04abc	3.15 $\pm$ 0.2b	3.78 $\pm$ 0.16b	2667.88 $\pm$ 61.93a	13.26 $\pm$ 0.99b	133.6 $\pm$ 7.19b	2443.84 $\pm$ 186.33b
		10 kJ/kg	1251.37 $\pm$ 71.98ab	4.8 $\pm$ 0.59b	1.78 $\pm$ 0.53bc	4.01 $\pm$ 0.47c	4.46 $\pm$ 0.34b	3219.41 $\pm$ 129.39b	18.69 $\pm$ 1.33c	165.1 $\pm$ 5.29c	3209.79 $\pm$ 178.05c

Means with different letters per variety are significantly different ($p < 0.05$)

Table 4 Concentration of selected terpenes ($\mu\text{g/L}$) of Grüner Veltliner and Traminer immediately after pressing (Must) and 3 months after bottling (Wine) related to different pretreatment and maceration time (mean value $\pm$ SD)

Variety	Macera- tion time (h)	Treatment	cis-Linalool oxide	trans-Linalool oxide	Lonalool	Hotrienol	Nerol oxide	alpha-Terpi- neol	Nerol	Citronellol	Geraniol
Must											
Grüner Velt- liner	4	Control	0.7 ± 0.23ab	0.38 ± 0.23ab	2.10 ± 0.32a	8.11 ± 0.63a	n.d	1.93 ± 0.19ab	0.84 ± 0.48a	0.48 ± 0.21b	n.d
		3 kJ/kg	0.57 ± 0.05a	0.28 ± 0.09ab	1.67 ± 0.13a	8.16 ± 0.43a	n.d	1.76 ± 0.22a	0.39 ± 0.13a	0.24 ± 0.05a	n.d
		10 kJ/kg	0.55 ± 0.08a	0.22 ± 0.02a	1.90 ± 0.06a	8.47 ± 1.11a	n.d	1.78 ± 0.14ab	0.42 ± 0.1a	0.22 ± 0.03a	n.d
	24	Control	1.29 ± 0.31c	0.59 ± 0.03b	3.27 ± 0.86b	15.84 ± 3.59b	n.d	2.39 ± 0.4ab	1.15 ± 0.61a	0.26 ± 0.11ab	n.d
		3 kJ/kg	0.94 ± 0.14bc	0.37 ± 0.07ab	3.11 ± 0.4b	14.15 ± 2.48b	n.d	2.16 ± 0.37ab	0.82 ± 0.17a	0.23 ± 0.05a	n.d
		10 kJ/kg	1.02 ± 0.11bc	0.32 ± 0ab	3.29 ± 0.31b	13.92 ± 0.78b	n.d	2.38 ± 0.29b	0.69 ± 0.16a	0.20 ± 0.05a	n.d
Traminer	4	Control	4.88 ± 0.76ab	3.22 ± 0.48b	32.31 ± 3.74a	29.16 ± 3.17ab	2.46 ± 0.26a	15.39 ± 0.8a	87.09 ± 2.68a	30.77 ± 5a	423.54 ± 20.06a
		3 kJ/kg	3.90 ± 0.52a	2.46 ± 0.25ab	39.38 ± 4.49a	31.58 ± 3.63abc	2.92 ± 0.03a	17.54 ± 1.86a	116.29 ± 15.17b	34.01 ± 5.1a	543.21 ± 82.05ab
		10 kJ/kg	3.65 ± 0.36a	2.19 ± 0.23a	32.11 ± 2.37a	24.15 ± 2.74a	2.52 ± 0.32a	13.53 ± 0.86a	99.92 ± 4.36ab	28.29 ± 2.97a	482.60 ± 39.02a
	24	Control	5.36 ± 0.76ab	3.04 ± 0.47ab	58.05 ± 6.44b	38.82 ± 5bc	4.28 ± 0.33b	27.56 ± 3.06b	174.64 ± 18.82c	42.87 ± 4.63b	666.55 ± 80.03b
		3 kJ/kg	8.98 ± 1.84c	4.67 ± 0.79c	95.10 ± 3.3c	61.13 ± 10.34d	6.00 ± 0.04c	49.18 ± 4.97d	292.02 ± 12.9e	68.47 ± 1.15d	1062.85 ± 67.22d
		10 kJ/kg	6.06 ± 0.61b	3.25 ± 0.32b	68.54 ± 7.06b	41.45 ± 4.35c	4.69 ± 0.11b	34.23 ± 3.71c	234.34 ± 15.32d	54.76 ± 2.22c	865.77 ± 80.41c
Wine											
Grüner Velt- liner	4	Control	0.64 ± 0.16d	0.31 ± 0.16ab	3.32 ± 0.38b	14.87 ± 3.28b	n.d	0.24 ± 0.05bc	0.31 ± 0.07a	6.19 ± 1.22b	n.d
		3 kJ/kg	0.47 ± 0.06ab	0.21 ± 0.05ab	2.87 ± 0.33ab	11.07 ± 1.13ab	n.d	0.16 ± 0.03a	0.32 ± 0.04a	5.10 ± 0.79ab	n.d
		10 kJ/kg	0.36 ± 0.04a	0.24 ± 0ab	2.48 ± 0.22a	9.00 ± 0.60a	n.d	0.16 ± 0.04ab	0.34 ± 0.06a	4.41 ± 0.44a	n.d
	24	Control	0.53 ± 0.01 cd	0.20 ± 0.03ab	3.18 ± 0.36ab	13.28 ± 1.4b	n.d	0.25 ± 0.03c	0.37 ± 0.05a	4.43 ± 0.12a	n.d
		3 kJ/kg	0.45 ± 0.04ab	0.16 ± 0.05a	2.90 ± 0.51ab	10.97 ± 2.21ab	n.d	0.15 ± 0.03a	0.37 ± 0.05a	4.32 ± 0.73a	n.d
		10 kJ/kg	0.39 ± 0.04ab	0.35 ± 0.06b	2.67 ± 0.03ab	9.12 ± 0.39a	n.d	0.19 ± 0.03abc	0.35 ± 0.04a	3.91 ± 0.33a	n.d
Traminer	4	Control	3.26 ± 0.26b	1.75 ± 0.07b	19.08 ± 1.45b	47.10 ± 2.65b	2.60 ± 0.24a	0.63 ± 0.02bc	19.96 ± 4.1a	48.54 ± 5.33a	58.08 ± 9.04a
		3 kJ/kg	2.62 ± 0.06a	1.23 ± 0.07a	14.31 ± 1.01a	37.14 ± 2.7a	2.28 ± 0.22a	0.40 ± 0.12a	18.52 ± 2.27a	39.64 ± 4.64a	49.92 ± 5.82a
		10 kJ/kg	2.85 ± 0.31ab	1.86 ± 0.23b	16.71 ± 1.17ab	45.10 ± 1.06b	2.81 ± 0.31a	0.49 ± 0.01ab	22.75 ± 4.3a	52.40 ± 3.16a	61.67 ± 10.36a
	24	control	3.98 ± 0.4c	1.97 ± 0.14bc	24.42 ± 1.52c	58.40 ± 5.2 cd	4.39 ± 0.2b	0.81 ± 0.12d	36.12 ± 1.99b	70.06 ± 5.2b	83.64 ± 2.76b
		3 kJ/kg	3.85 ± 0.06c	2.14 ± 0.07c	23.90 ± 0.66c	54.71 ± 2.77c	4.30 ± 0.32b	0.69 ± 0.04 cd	42.69 ± 4.8b	75.36 ± 6.42b	99.40 ± 7.19b
		10 kJ/kg	4.59 ± 0.27d	2.21 ± 0.09c	26.49 ± 2.30c	64.34 ± 3.22d	4.82 ± 0.12b	0.85 ± 0.11d	46.46 ± 8.58b	79.21 ± 13.84b	103.39 ± 17.22b

Means with different letters per variety and storage time are significantly different ($p < 0.05$)

Fig. 4 Content of the sesquiterpene rotundone of Grüner Veltliner in dependency of the applied pretreatment and maceration time. Means with different letters are significantly different ($p < 0.05$)

to the enzyme treated control was found immediately after pressing performed after 24 h maceration time. However, the beneficial effect of PEF was not detectable anymore 3 months after bottling. Moio et al. [42] already described that during the fermentation process, volatile compounds such as bound geraniol, benzyl alcohol and 2 phenylethanol significantly decrease and in some cases free forms of linalool and benzyl alcohol increase by fermentation.

Moreover, the sensory panel detected no significant differences between the tested pretreatments based on the 20 point scheme. All samples originating from the same variety were described by the same values (results are not shown). Furthermore, the evaluation of the descriptive analysis did not result in any specific assignments of sensory attributes. Hence, although relevant volatile compounds especially of the variety Traminer got increased by the application of PEF, the sensory evaluation did not show detectable benefits related to the olfactory character of the wine.

These results are also in correspondence with the findings from Puértolas et al. [11]. They detected a positive impact of PEF on colour intensity, anthocyanin content, total polyphenol and tannin content of Cabernet Sauvignon. However, sensory assessment after 4 months bottle ageing resulted in similar results for the pretreated and control wines.

Conclusion

The present study shows that selected primary aroma compounds, which are mainly located in the mesocarp, can be extracted more efficiently due to the increased cell permeabilisation, based on a PEF pretreatment. In contrast to previous works, no beneficial effects of PEF on juice yield were detectable for the given experimental setup considering

the use of conventional pectolytic enzymes and the applied pressing system. The PEF induced increase of released nitrogen can lead to a reduction of the mean fermentation time of up to 20% depending on the variety. The release of compounds associated to the skin, such as rotundone, were not affected by the application of PEF. Furthermore, adverse outcomes of the PEF treatment were identified related to the increase of turbidity and the higher amount of polyphenols which are undesired effects especially for white wine. However, it was detectable that the advantages of PEF in combination with the application of maceration enzymes significantly depends on the processed variety. Especially for the production of wines with specific aroma profiles, such as Traminer and Muscat, the application of electric fields is very promising. To conclude on the benefits from the use of PEF for white wine production, further varieties and processing parameters at the different steps of the vinification process need to be investigated.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethical requirements This article does not contain any studies with human or animal subjects.

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