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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1693733 since 2019-02-28T11:22:47Z

Published version:

DOI:10.1016/j.foodres.2019.02.034

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(Article begins on next page)

28 February 2024

Accepted Manuscript

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PII: S0963-9969(19)30120-6
DOI: https://doi.org/10.1016/j.foodres.2019.02.034
Reference: FRIN 8294

To appear in: *Food Research International*

Received date: 31 August 2018
Revised date: 15 February 2019
Accepted date: 19 February 2019

Please cite this article as: S. Giacosa, M. Gabrielli, F. Torchio, et al., Relationships among electrolyzed water postharvest treatments on winegrapes and chloroanisoles occurrence in wine, Food Research International, https://doi.org/10.1016/j.foodres.2019.02.034

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Relationships among electrolyzed water postharvest treatments on winegrapes and chloroanisoles occurrence in wine

Simone Giacosa[a#], Mario Gabrielli[b#], Fabrizio Torchio[a,c], Susana Río Segade[a]*, Ana Maria Moar Grobas[a], Davide Ricauda Aimonino[a], Paolo Gay[a], Vincenzo Gerbi[a], Chantal Maury[b], Luca Rolle[a]

[a] *Università degli Studi di Torino. Dipartimento di Scienze Agrarie, Forestali e Alimentari. Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy.*

[b] *USC 1422 GRAPPE, INRA, Ecole Supérieure d'Agricultures, SFR 4207 QUASAV, 55 rue Rabelais, 49100 Angers, France.*

[c] *Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy.*

[#] These authors contributed equally to the study.

* Corresponding author. E-mail: susana.riosegade@unito.it

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Abstract

Electrolyzed water (EW) has attracted much recent attention as a high-performance, new technology for its potential use in the food industry. However, the risk of postharvest EW treatments of winegrapes destined for winemaking is the chloroanisoles formation in the final product. In the present study, we investigated the impact of postharvest grape EW and storage treatments on the occurrence of these compounds in wine, 2,4,6-trichloroanisole (TCA) being the main compound responsible for the cork taint off-flavor due to its extremely low perception threshold. The results revealed that the presence of TCA in the wines depended on the concentration of residual free chlorine in the must from the EW treatment. Particularly, TCA was not detected in wine when active chlorine concentrations higher than 0.005 mg/L were present in the must. Chloroanisole and chlorophenol levels in wine were strongly affected not only by EW but also by grape storage conditions (temperature, time, sunlight exposition). The results showed that the 24-hours grape storage at a controlled temperature of 20 °C in the dark, after EW treatment, resulted in the highest TCA concentrations in wines (7.3 ± 2.7 ng/L), while storage and withering in sunlight seemed to mitigate its presence in wine. This could suggest that microbiological formation of chloroanisoles may have been influenced by the storage temperature and germicidal effect of UV light. Biochemical mechanism of chloroanisoles production in grapes and wine is unknown, but the combination of residual free chlorine on the grape surface and the controlled storage conditions in the dark may have positively influenced the bio-formation of chloroanisoles and therefore their presence in wine.

Keywords: grapes, electrolyzed water, cork taint, chloroanisoles, postharvest treatments, storage conditions.

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1. Introduction

Electrolyzed water (EW) is an environmentally friendly and emerging antimicrobial sanitizer (Huang, Hung, Hsu, Huang, & Hwang, 2008). EW is produced by electrolysis of dilute solutions of NaCl (also KCl or $MgCl_2$) in an electrolysis cell with or without diaphragm, which separates the anode and cathode (Al-Haq, Sugiyama, & Isobe, 2005). When the electrolysis chamber is divided by a septum, two types of EW are produced: acid- (AcW) and alkaline-electrolyzed waters (AlW) (Liao, Chen, & Xiao, 2007). AcW has pH < 2.7 and oxidation-reduction potential (ORP) between 1000 and 1100 mV, while AlW shows pH > 10 and ORP between −450 and −1000 mV (Hsu, 2005). On the contrary, in an electrolysis cell without membrane, neutral electrolyzed water (NEW) is obtained. NEW exhibits pH ≈ 7 and ORP around 750 mV (Deza, Araujo, & Garrido, 2007). NEW has been also produced using separating membranes with pH ranging from 5.0 to 8.5 and ORP between 700 and 900 mV (Graça, Abadias, Salazar, & Nunes, 2011). AcW and NEW are particularly employed for the disinfection and preservation of foodstuffs (Issa-Zacharia, Kamitani, Muhimbula, & Ndabikunze, 2010), while AlW is also used for removing dirt and grease from several materials (Hsu, 2005) but it leaves chlorine residues on the treated surfaces (Guentzel, Lam, Callan, Emmons, & Dunham, 2010).

EW shows antimicrobial action against a broad spectrum of microorganisms, including bacteria, mold and yeast, mainly due to its content of free chlorine (Huang et al., 2008). The chlorine species (Cl_2, ClO^-, and HClO) are strongly affected by the pH of EW (Hricova, Stephan, & Zweifel, 2008). Hypochlorous acid (HClO) shows a higher sanitizing power than hypochlorite ions (ClO^-) when the pH of EW ranges between 5.0 and 6.5 (Cao, Zhu, Shi, Wang, & Li, 2009). HClO acid can penetrate the microbial cell membranes and subsequently produces hydroxyl radicals ($OH^{\cdot}$), which exert antimicrobial action through the oxidation of key metabolic systems (Albrich, Gilbaugh, Callahan, & Hurst, 1986; Hurst, Barrette, Michel, &

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Rosen, 1991). Instead, at pH values ≈ 9.5, the effectiveness of sanitizing treatment is related to the combined action of OH^-, ClO^-, and H_2 gas nanobubbles (Lyu, Gao, Zhou, Zhang, & Ding, 2018). These nanobubbles can improve the activity of AlEW by acting on the particulates present on the treated surface.

For some of these reasons the use of EW in food industry has increased significantly (Hricova et al., 2008; Huang et al., 2008). Several studies have reported that EW is effective in reducing the growth of different microorganisms during the postharvest storage of many fruit and vegetables (Al-Haq, Seo, Oshita, & Kawagoe, 2002; Guentzel et al., 2010; Guentzel, Callan, Lam, Emmons, & Dunham, 2011; Okull & Laborde, 2004; Whangchai, Saengnil, Singkamanee, & Uthaibutra, 2010). In fresh winegrapes, this sanitizing agent has been also used to select mycobiota present, and even to reduce the possible presence of *Brettanomyces bruxellensis*, on the berries surface and therefore to improve the wine quality (Cravero et al., 2016; Cravero et al., 2018).

When considering the wine industry, the excessive use of chlorine-based cleaning solutions in winery was related to the formation of chloroanisoles (Simpson & Sefton, 2007), which are the main compounds responsible for corked off-odor in wine (Callejón, Ubeda, Ríos-Reina, Morales, & Troncoso, 2016). Cork taint off-flavor is one of the major organoleptic defects in wine, and it is commonly associated with a musty or moldy aroma (Prescott, Norris, Kunst, & Kim, 2005). The main compounds responsible for "cork taint" in wine are: 2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA), pentachloroanisole (PeCA) and 2,4,6-tribromoanisole (TBA) (Cravero, Bonello, Pazo Alvarez, Tsolakis, & Borsa, 2015; Evans, Butzke, & Ebeler, 1997). Among the chloroanisole compounds, TCA is the most powerful off-flavor (Callejón et al., 2016) because its perception threshold is extremely low ranging from 0.03 to 1-2 ng/L in water (Griffith, 1974), 2 to 10 ng/L in white wine and 5 to 22 ng/L in red wine (Alvarez-Rodriguez et al., 2002; Prescott et al., 2005). Most studies suggest that

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filamentous fungi (primarily *Penicillium* spp., followed by *Aspergillus* spp., *Cladosporium* spp. *Trichoderma* spp. and *Fusarium* spp. *Botrytis* spp.) are the main microorganisms producing these undesirable metabolites, but some authors pointed out also the implication of species of different yeast genera (Bureau, Charpentier-Massonnat, & Pansu, 1974; Prak, Gunata, Guiraud, & Schorr-Galindo, 2007). The factors thought to contribute to the accumulation of chloroanisoles in cork, wine and food have been reviewed by several authors (Lee & Simpson, 1993; Mottram, 1998; Pereira, Figueiredo Marques, & San Romao, 2000). All proposed that microbiological formation pathway of chloroanisoles involves the methylation of chlorophenols (Prak et al., 2007). This transformation is a detoxification mechanism whereby microorganisms remove chlorophenols from environment (Tindale, Whitfield, Levingston, & Nguyen, 1989). However, the microorganisms reported above were frequently isolated from grapes and cellar environment (Barata, Malfeito-Ferreira, & Loureiro, 2012; Goto, Takayama, & Shinohara, 1989). Haas et al. (2010) have identified 35 fungal genera and 84 species in the air, on walls and barrels of different wineries.

In the wine cellar, halogenated phenols can be introduced through different sources, such as wooden pallets, cartons, packing materials and the use of hypochlorite solutions in the cleaning of wooden barrels (Callejón et al., 2016). Several authors have reported that the chlorine contamination of organic materials in contact with wine may lead to the risk of the chloroanisoles formation by direct chlorination of phenols followed by bio-methylation (Burttschell, Rosen, Middleton, & Ettinger, 1959; Karlsson, Kaugare, Grimvall, Boren, & Savenhed, 1995; Simpson & Sefton, 2007). Molds and yeasts could contribute to the bio-methylation of chlorophenols and therefore to the presence of chloroanisoles in wine.

Despite the fact that the antimicrobial effectiveness of EW during the postharvest storage of a wide variety of fruit and vegetables has been demonstrated, its use in the wine industry is unknown until now. EW could be an interesting alternative to reduce the presence

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of undesirable yeast and fungi species on grapes. However, to our knowledge, in literature there are no data available about the effect of grape postharvest EW treatments on the presence of chloroanisoles in wine. Therefore, the main purpose of this work was to investigate the risks of using EW for sanitizing postharvest grapes destined for winemaking, as well as to study if short- and long-term grape storage can induce changes in the chloroanisoles occurrence in the wine. With this aim, the influence of residual free chlorine concentration in the must after the grape treatment with EW on the presence of chloroanisoles in wines was evaluated. Additionally, this work examined the impact of different storage time (fresh and partially dehydrated winegrapes) and conditions (temperature and solar radiation) of EW-treated grapes on the occurrence of chloroanisoles in red wines after four months from the end of alcoholic fermentation.

2. Materials and Methods

2.1. Chemicals

Chemical reagents and analytical standards were provided by Sigma-Aldrich (St. Louis, MO, USA). Deionized water was produced by a DEIONEX TWO system (Appen.Lab, Torino, Italy), while standards were prepared in ultrapure water produced using a Milli-Q system (Merck Millipore, Darmstadt, Germany).

2.2. Grape samples

Vitis vinifera L. cv. Barbera red winegrapes were harvested at ripeness from a vineyard located in Alba (Cuneo province, northwest Italy) in 2014. At harvest, the grape must evaluated using OIV (2012) methods evidenced a soluble solids content of 25.2°Brix, a pH value of 3.22, and a titratable acidity of 9.4 g/L as tartaric acid. Once in the laboratory, the clusters were cut to form small groups of 3–5 berries with pedicel attached, and thereby used in the subsequent trials summarized in **Figure 1**.

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2.3. Electrolyzed water production

Electrolyzed water (EW) solution was freshly produced using a EVA SYSTEM® 100 equipment (Industrie De Nora S.p.A., Milan, Italy) following the electrolysis procedure described by Cravero et al. (2016). The EW solution obtained contained 4,000 mg/L of free chlorine at ≈ pH 9. Free chlorine concentration was determined by iodometric titration (APHA, 1992). This solution was diluted with deionized water to obtain 400 mg/L of free chlorine solution, which was used in the experiment either for adding to the must in the evaluation of the effect of possible free chlorine remaining after grape treatment or for intact grape treatment, as described below.

2.4. Residual free chlorine in grape must

To simulate the effect of free chlorine remaining in the must after grape EW treatment, a direct addition of increasing EW volumes was done on the must before fermentation. To this aim, 5 L of must were produced by crushing untreated fresh Barbera grapes. Only the liquid must was kept, discarding the solid parts (skins and seeds). The must was homogenized by stirring at 25 °C temperature and inoculated with 200 mg/L of rehydrated Uvaferm BC yeast (Lallemand Inc., Montreal, Canada). Then, in each of 18 flasks, 250 mL of the previously inoculated must were immediately introduced and the volume required of EW was added to achieve different free chlorine concentrations (nine concentrations, in duplicate): 0 (control, no addition), 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 1 and 10 mg/L (samples code M). Alcoholic fermentation was performed at 25 °C for 14 days, and then the wine was racked and stored in closed glass containers at 10 °C for 4 months before analysis. A schematic representation of this experiment was shown in **Figure 1**.

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2.5. Whole grape berry treatments with electrolyzed water

All EW treatments were carried out in duplicate. For each treatment and replicate, 500 g of the previously prepared grapes in small groups of 3–5 berries were placed, in a single layer, in zinc-coated steel containers with meshes of 8 ×8 mm, designed to avoid stagnating water/solutions and to let air flow through berries during the following phases (Rolle, Caudana, Giacosa, Gerbi, & Río Segade, 2011). Except for the control sample (CW), which was used for winemaking "as is", each berry group was completely immersed in the EW solution (400 mg/L of free chlorine) for 1 min and then removed from it. No rinsing was carried out after treatment, except for the "rinsed" experiment. In this latter case, the grapes after EW treatment were rinsed by immersion in deionized water for 1 min, left to drain off for 1 min, and then immediately used for winemaking (W^{0h} sample).

After EW treatment, berry groups were separately stored in the previously described metallic containers: (a) in the dark for 24 h at 10 °C ($W^{10°C}$), 20 °C ($W^{20°C}$) and 30 °C ($W^{30°C}$); or (b) outside, protected from the rain and west solar exposed, in a storage/dehydration trial. In this latter case, the storage period started at noon and lasted 4 h (W^{4h}) and 24 h (W^{24h}). Furthermore, longer exposure times were evaluated to conduct grape dehydration, such as 96 h (W^{96h}), 168 h (W^{168h}) and 500 h (W^{500h}). The schematic representation of this experiment can be seen in **Figure 1**. Solar radiation and temperature were registered during the whole experiment as described in the next sections. The weight loss was calculated as the ratio between the weight of dehydrated and fresh samples, and expressed in percentage. Weight was determined using a technical balance with a precision of 0.01 g (Gibertini E1700, Modena, Italy).

At the end of the storage/dehydration trials, the grape samples were subjected to lab-scale winemaking. Each grape sample was manually destemmed and crushed to obtain at least 250 mL of grape mash (must plus skins), which were placed in wide-neck Erlenmeyer flasks.

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In this experiment, the treated skins were kept and macerated during fermentation to evaluate the effect of grape surface EW treatments. Alcoholic fermentation started immediately after crushing with the inoculum of 200 mg/L of rehydrated Uvaferm BC yeast (Lallemand Inc.), and progressed at 25 °C for 14 days. Then, the macerated skins were removed, and the wine was racked and stored in closed glass containers at 10 °C for 4 months before analysis.

2.6. Measurement of temperature and solar radiation during grape storage and withering

Temperature and global solar irradiance incident on bunches were monitored every 5 min during the entire process (500 h). Berries surface temperature was measured by five T-type thermocouples (0.6 mm diameter and 10 mm long junctions) connected to a Data Taker D65 (Thermo Fisher Scientific, Scoresby, Victoria, Australia) data logging system and arranged among the berries of as many bunches, avoiding the direct sun exposure of the junctions. Global solar irradiance was measured by a Delta OHM LP471 RAD sensor (400−1050 nm spectral range with cosine correction diffuser; Delta OHM, Caselle di Selvazzano, Italy) connected to a Delta OHM DO 9847 data logger and synchronized with the previous one. The irradiance sensor was positioned close to the grid where bunches were arranged, parallel to the grid plane and at the same height.

2.7. Temperature and radiation indexes

The Time Temperature Integral (*TTI*) and Global Solar Radiation (*GSR*) were calculated in order to monitor the temperature and solar radiation of the storage/dehydration process. Let $\bar{T}$ the average of the temperatures recorded by the five thermocouples and $\bar{T}_{min}$ the absolute minimum value of the average temperature recorded during the withering process, the TTI was calculated at every sample time (t^*) by the equation:

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$$TTI = \int_0^{t^*} \bar{T}(t) - \bar{T}_{min}\, dt \quad (1)$$

where the integral was numerically computed by the trapezoidal rule, while sampling times were $t^* = \{0, 4, 24, 96, 168, 500\}$ hours.

Likewise, the GSR, which represents the amount of solar energy incident on a unit area, was computed, at every sample time (t^*), as the integral over the time of the global solar irradiance (I):

$$GSR = \int_0^{t^*} I(t)\ dt \quad (2)$$

2.8. Wine analysis: residual sugars, ethanol, chloroanisoles and chlorophenols contents

The wines obtained were analyzed by HPLC to assess the residual sugars content and the ethanol produced by yeasts, using a 1260 HPLC-DAD-RID system (Agilent Technologies, Santa Clara, CA, USA) and following the method described by Rolle et al. (2018).

Each wine sample was analyzed for its content in chloroanisoles and chlorophenols using headspace solid phase microextraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS) technique. Chloroanisoles determination followed ISO 20752:2014 (ISO, 2014) procedure using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C quadrupole mass detector (Agilent Technologies). For chlorophenols determination, the same equipment was used with the following procedure: 0.9 g of NaCl, 2.5 mL of water, 0.5 mL of H_2SO_4 1 mol/L solution and 2 mL of wine were added in a 20 mL glass vial, and spiked with 50 µL of an internal standard solution (50 µg/L of 2,3,6-trichloroanisole in methanol). The vial was kept at 25 °C temperature under stirring, a 85 µm polyacrilate SPME fiber (Supelco,

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Bellefonte, PA, USA) was exposed to the vial headspace for 20 min, and then the analytes were desorbed from the fiber in the GC system for 3 min at 280 °C under splitless conditions. For the chromatographic separation, a HP-5 capillary column (crosslinked 5% PHME siloxane, 30 m × 0.25 mm i.d. × 0.25 μm) was used under the following temperature program: 50 °C isothermal temperature for 4 min, 12 °C/min ramp to 210 °C, 20 °C/min ramp to 300 °C final temperature, and then the latter kept for additional 10 min. The detected compounds by GC-MS, their abbreviation and limit of detection (LOD) were: 2,4,6-trichloroanisole (TCA, LOD 0.5 ng/L), 2,3,4,6-tetrachloroanisole (TeCA, LOD 0.5 ng/L), pentachloroanisole (PeCA, LOD 1 ng/L) and 2,4,6-trichlorophenol (TCP, LOD 1 ng/L). The analytical method used allowed also the detection of 2,3,4,6-tetrachlorophenol, pentachlorophenol, 2,4,6-tribromoanisole, 2,4,6-tribromophenol, 2,3,4,6-tetrabromophenol, 2-methylisoborneol, geosmin (all with LOD 1 ng/L) and pentabromophenol (LOD 5 ng/L). However, these latter compounds were not found in the analyzed samples, with two exceptions described in the following sections. Results were expressed as ng of the determined compound (calculated through calibration curves considering the ratio between the compound and the internal standard area) per liter of wine.

2.9. Statistical analysis

Analysis of variance (ANOVA) and post-hoc Tukey-b tests were carried out using the SPSS Statistics suite (IBM Corporation, Armonk, NY, USA) version 25. Given the limited number of replicates, for statistical analysis, the data below the limit of detection ("non-detects") were considered as half value of the limit of detection (LOD) for each compound determined (EPA, 2000).

3. **Results**

3.1. Environmental conditions and grape dehydration process

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Environmental conditions during the storage/dehydration process of grapes are shown in **Figure S1** for temperature and in **Figure S2** for solar radiation. During the first 96 h, the weather was generally sunny (**Figures S1a and S2a**). Maximum temperature and solar radiation ranged 27-29 °C and 420-530 W/m^2, respectively. Over the following 214 h (96-310 h period), with the exception of the afternoon of day 5, the maximum temperature and solar radiation decreased to 17-20 °C and 30-70 W/m^2, respectively, achieving very low ΔTTI and ΔGSR values between 96 and 168 h (**Table 1**). During the natural grape withering in sunlight, the highest GSR and TTI values were recorded from 0th to 110th h and from 310th to 490th h.

Grape dehydration reached about 10% of weight loss in the first 96 h. This percentage increased until about 12% after 168 h of dehydration, evidencing a very slow dehydration trend between 96 and 168 h due to the decrease of temperature and especially of solar irradiance (**Figures S1a and S2a**; **Table 1**) as previously shown. Grape dehydration continued until 500 h at which time about 26% of total weight loss was reached.

3.2. Residual free chlorine in grape must

All the wines made from must added with free chlorine using EW reached residual sugar contents less than 4 g/L and ethanol contents ranged from 12.8 to 13.5% v/v. The impact of the presence of free chlorine in the must on the chloroanisoles and chlorophenols levels in the wine is reported in **Table 2**. As can be observed, 2,4,6-trichlorophenol (TCP) and pentachloroanisole (PeCA) were found more frequently in higher quantities than 2,3,4,6-tetrachloroanisole (TeCA) and 2,4,6-trichloroanisole (TCA). PeCA was detected in all added samples, although its concentration did not differ among treatments, while TeCA was only found in the wines resulting from the addition of 0.01 and 0.05 mg/L of free chlorine. The must spiked with the lowest amounts of free chlorine (between 0.0005 and 0.005 mg/L) showed TCA in wine, but TCA was not detected when free chlorine exceeded 0.005 mg/L. On the contrary, the

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concentration of TCP increased when the highest levels of free chlorine were added to the must (1 and 10 mg/L), even if the highest TCP value was observed at 0.0005 mg/L of free chlorine.

3.3. Grape 24-hours storage in the dark at three different temperatures (10, 20 and 30 °C)

All wines obtained from grape berry samples kept at controlled temperature for 24 h in the dark, after EW treatment, showed residual sugar concentrations less than 4 g/L. Nevertheless, a slight increase of the ethanol concentration occurred in the samples kept at 30 °C (average 14.3% v/v) and 20 °C (14.2% v/v) with respect to the samples kept at 10 °C temperature (13.8% v/v), presumably caused by the modest loss of water during storage at 20 and 30 °C temperatures. **Table 3** shows the levels of chloroanisoles and chlorophenols in wine produced from fresh grapes, treated with EW and then stored for 24 h in the dark at three different temperatures (10 °C, 20 °C, 30 °C). Grape treatment step with EW tended to increase the concentrations of TCA, PeCA and TCP compared to control. Despite the high variability of results, EW treatment followed by grapes 24-h storage resulted in a slight increase of TCA when compared to W^{0h} sample (washed and rinsed). TCA concentration was significantly higher (p<0.05) only in $W^{20°C}$ (7.3 ng/L) than in control (no EW treatment, no storage). TeCA was only found in wine $W^{20°C}$ at 0.5 ng/L, while TCP slowly decreased with the temperature from $W^{10°C}$ to $W^{30°C}$. In one replicate of $W^{10°C}$ sample, 2,3,4,6-tetrachlorophenol (TeCP) was also detected at 11 ng/L.

3.4. Grape storage in the sunlight (0, 4 and 24 h)

Must fermentations followed similar performances in all grape samples (control or treated with EW), which were completed within 14 days giving wines with residual sugar levels < 4 g/L and ethanol contents of ≈ 14% v/v. **Table 4** shows the chloroanisoles and chlorophenols

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content for wines obtained from grapes treated with EW and subsequently stored in sunlight for 4 and 24 h. TCA, PeCA and TCP were detected in the treated samples, while TeCA was not found in any wine. During short-term grape sunlight storage few differences were observed in chloroanisoles contents with respect to control or non-sunlight exposed samples, as well as between 4 and 24 h sunlight stored samples, for each compound. The highest concentration of TCA (3.7 ng/L) was reached in W^{4h} wine followed by W^{0h} (2.6 ng/L), which were significantly higher than that corresponding to control (p<0.05). However, longer storage times (W^{24h}) decreased slightly the presence of TCA. Among chloroanisoles and chlorophenols, the highest concentrations were found for TCP in all wines made from EW-treated grapes. Sunlight storage led to significantly lower amounts of TCA (p<0.05) in wines than storage in the dark, while PeCA and TCP showed no differences between these two different storage conditions. In addition, pentachlorophenol (PCP) was only found in one replicate of W^{24h} sample, which achieved 29 ng/L.

3.5. Grape withering in the sunlight (96, 168 and 500 h)

Wines made from dehydrated grapes (96-500 h) had higher residual sugars and ethanol contents than those produced from 0-24 h stored samples, reaching respectively ≈ 16 g/L and 15.0% v/v for W^{96h}, 9 g/L and 15.7% v/v for W^{168h}, 55 g/L and 15.9% v/v for W^{500h}. **Table 4** shows the effect of grape EW and sun-dehydration treatments on the occurrence of chloroanisoles and chlorophenols in wines. TCA, PeCA and TCP were detected in the treated samples whereas TeCA was never detected. The increase of grape dehydration time did not affect the level of chloroanisoles (p>0.05) in the resulting wines as a consequence of the high variability in the results obtained, although TCA and PeCA achieved the highest concentrations (2.3 and 16.5 ng/L, respectively) at the end of withering process. Furthermore, the lowest concentration of TCA was found in the wine obtained from grapes dehydrated for 168 h in

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sunlight, when the lowest ΔGSR and ΔTTI values were recorded. TCP did not differ significantly among the different withering stages (p>0.05), although its concentration in the wine declined from 61.5 to 38.0 ng/L when storage progressed from 168 to 500 h, respectively.

4. Discussion

To our knowledge, this study is the first attempt at describing the effect of grape treatments with electrolyzed water (EW), at different storage conditions before winemaking, on the occurrence of chloroanisole compounds in wines. The results showed that grape storage conditions after postharvest EW treatment played an important role in developing TCA in wine. Untreated samples (control wine, CW), in all experimental conditions, did not exhibit any presence of both chlorophenol and chloroanisole compounds. Therefore, the EW solution used for grape treatment was the only source of chloroanisoles.

The appearance of moldy and musty off-flavors in wines and fruit is related to microbial contaminants, chemical reactions within the commodity and airborne contaminants (Haas et al., 2010; Hui, 2010; Reineccius, 1991). Several fungal strains isolated from grape and cellars showed also the ability to produce TCP, direct precursors of TCA, from lignin degradation products and chlorinated compounds (Fontana, 2012; Pizarro, Pérez-Del-Notario, Sáenz-Mateo, & González-Sáiz, 2014). TCA is produced through the methylation of 2,4,6-trichlorophenol (TCP) by several microorganisms isolated from fruit, cork and wine cellar (Callejón et al., 2016; Haas et al., 2010; Whitfield, Nguyen, & Tindale, 1991). In the present study, TCA was found in the wines obtained at concentrations below its perception threshold, except in $M^{+0.005}$ sample where it reached 18.8 ng/L. Olfactory threshold of TCA is considerably impacted by the age, style and variety of wine (Mazzoleni & Maggi, 2007), and its presence is considered a defect in concentrations close to 10 and 40 ng/L in white and red wines, respectively (Riu, Mestres, Busto, & Guasch, 2002). Therefore, it is important to know the most

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influential parameters in the possible presence of TCA in wines after using EW as grape sanitizing treatment.

On the one hand, free chlorine remaining in the must after the grapes EW treatment influenced the presence of chloroanisoles in the resulting wines. TCA was not detected in samples spiked with the highest amounts of active chlorine, where TCP reached the higher concentrations. These results indicated that high amounts of residual free chlorine could have inhibited the bio-formation of TCA. This could be due to the antimicrobial effect of EW on the germination of different fungal conidia (Buck, 2002), although the complete inactivation of fungi requires EW with residual chlorine levels ≥ 10 mg/L. Furthermore, the results reported by Zhu et al. (2016) showed that the use of EW with pH > 6.5 or even alkaline EW during the winemaking of persimmon caused the suppression of certain yeast activities during alcoholic fermentation, such as those involved in the conversion of amino acids into alcohols and esters, without compromising the complete transformation of sugars into ethanol.

On the other hand, the effectiveness of sanitization and the appearance of TCA in wine could be also strongly affected by grape storage conditions after postharvest EW treatment. In fact, several research studies reported that EW was able to reduce the initial microbial loads of fruit and vegetable, but its microbial inactivation disappeared after storage under controlled conditions (Chiabrando, Peano, & Giacalone, 2017; Guentzel et al., 2010; Jeong, Kim, Kwon, & Park, 2006; Martínez-Hernández, Artés-Hernández, Gómez, Formica, & Artés, 2013; Rahman, Yong-Guo, & Deog-Hwan, 2011; Whangchai et al., 2010). In our work, the sunlight storage and withering did not seem to influence strongly the chloroanisoles levels in wine. Nevertheless, 24-h storage in the dark at 20 °C led to significantly higher amounts of TCA (p<0.05) in wine than the storage and withering under natural environmental conditions. This may be related to the storage temperature and the germicidal effect of UV light, which are the main factors influencing germination, growth and sporulation of spoilage fungi (Magan &

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Lacey, 1984). The effect of environmental factors on the growth of different molds isolated from grapes (*Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp. and *Botritis* spp.) was studied by Judet-Correia et al. (2010), and they demonstrated that the isolate grew best at 20 °C, whereas at 10 °C and 30 °C it showed a sharp decline (Bellí, Marín, Duaigues, Ramos, & Sanchis, 2004). In addition, the results of Valero, Marín, Ramos and Sanchis (2005) showed that only few microorganisms are capable to resist the germicidal power of both UV light and the strong sunlight heating (Rotem & Aust, 1991).

During grape withering under direct sunlight, it was observed an opposite trend of TCA, PeCA and TCP between 168th and 500th h, which corresponded to the highest global solar radiance (GSR) recorded. At the end of dehydration process, TCA and PeCA reached the highest concentrations in wine whereas the lowest levels of TCP were observed. The decrease of TCP may be related to both its bio-transformation into chloroanisoles (Callejón et al., 2016) and its photodegradation by UV-irradiation. The photodegradation kinetics of chlorophenols in water solution was studied by Tamer et al. (2006) who demonstrated that the UV-irradiation treatment of a mixture of TCP and PCP (50 mg/L each) allowed the complete removal of chlorophenols after 40 h of UV-exposure. Boule, Guyon and Lemaire (1982), Kawaguchi (1992), and Tratnyek and Holgne (1991) reported that the photodegradation rate of chlorophenols depends on the structure form of the occurring compound and on the pH of the reaction environment. The chlorine atom at the 4- and 6- positions is photo-labile while it is not photoreactive at 3- and 5- positions (Kochany and Bolton, 1991). Therefore, depending on the molecule structure, the rate and yield of these processes may vary considerably.

EW at pH 9 contains primarily hypochlorite ions (ClO^-; 80–95%), which leave chlorine residue on fruit surfaces (Hricova et al., 2008). In our study, the detection of chloroanisoles in wines may suggest that the residual chlorine on grapes after EW treatment and storage under controlled temperature in the dark could have promoted the microbiological formation pathway

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of TCA and therefore its presence in wine. Spadone, Takeoka and Liardon (1990), and Liardon, Spadone, Braendlin and Denton (1989) reported interesting results on the formation of TCA by molds and bacteria isolated from infected beans of green coffee. Specifically, the microorganisms incubated in a media containing NaClO were able to produce quantities of TCA comparable to those detected in infected coffee beans in the presence of chlorine. Subsequent studies reported similar results when the fungi were grown on green coffee beans in presence of NaClO (Liardon, Braendlin, & Spadone, 1991). Moreover, Maujean, Millery and Lemaresquier (1985) also showed that the cultures of *Penicillium* spp., isolated from a contaminated cellar and Champagne cork, synthesized TCA in the presence of hypochlorite or chlorine. On the contrary, Aung, Smilanick, Vail, Hartsell and Gomez (1996)**,** and Aung and Jenner (2004) observed that TCA was formed in dried grapes (raisins) in absence of both microorganisms and chlorophenol compounds. They supposed that the grapes could bio-transform native phenols into chloroanisoles through methyltransferases present in *Vitis* berry skin tissues (Boss, Davis, & Robinson, 1996) and the use of chlorinating systems (Axelrod & Daly, 1968; Jerina, Guroff, & Daly, 1968). Furthermore, Jäger, Diekmann, Lorenz and Jakob (1996) evidenced the ability of cork-borne bacteria and yeasts to develop unpleasant aromas when inoculated in test models including cork granules and TCP in wine (11.5% v/v ethanol). Given the fact that postharvest grape EW treatments and storage conditions have caused the appearance of TCA, the mechanism of EW effects on the occurrence of chloroanisoles in wine should be studied further.

5. Conclusions

To our knowledge, this is the first study examining electrolyzed water and chloroanisoles formation in the production of wine. Postharvest grape EW treatments affected chloroanisoles and chlorophenols in wine, but their contents could be partially modified during

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grape storage before winemaking. The rinsing of the grapes after the EW treatment did not reduce the TCA contents in wines. Nevertheless, short-term fresh grape storage under uncontrolled conditions in sunlight mitigated the levels of TCA in wine by reducing the chlorine contamination from EW. The same effect was observed during grape withering and, if confirmed by further specific studies, it could be exploited for the production of dry and sweet wines with special aroma, natural sun-exposure being the most traditional and still widely used method for winegrapes dehydration in sunny Mediterranean regions. From a prudential point of view, EW should not be used for postharvest treatment of grapes destined for winemaking because of the possible risk of TCA occurrence in the resulting wines. These results constitute a basis for further studies focused on the antimicrobial effectiveness of EW against grapes molds infection in vineyard and its contribution to the appearance of TCA in wine.

Acknowledgments

This research was conducted in the framework of the regional programme "Objectif Végétal, Research, Education and Innovation in Pays de Loire", supported by the French Region Pays de la Loire, Angers Loire Métropole and the European Regional Development Fund". The authors also would like to thank Cristian Carboni (Industrie De Nora S.p.A., Milan, Italy) for the availability of the EW production system.

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FIGURE CAPTIONS

Figure 1. Schematic representation of the different experimental sets carried out.

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Table 1. Values of Time Temperature Integral (TTI) and Global Solar Radiation (GSR) calculated at each sampling time; ΔTTI and ΔGSR are the variation of TTI and GRS between two subsequent sampling times.

Storage – withering time	**TTI - Time Temperature Integral [°C h]**	**ΔTTI [°C h]**	**GSR - Global Solar Radiation [MJ m^{-2}]**	**ΔGSR [MJ m^{-2}]**
Short-term grape storage				
0 h	*0*	*0*	*0*	*0*
4 h	73	73	3.0	3.0
24 h	348	275	5.1	2.1
Grape withering				
96 h	1385	1037	17.4	12.3
168 h	2261	876	21.7	4.3
500 h	6764	4503	50.4	28.7

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Table 2. Concentration range of chloroanisoles and chlorophenols in wines after four months from the end of alcoholic fermentation, made from musts added with free chlorine using electrolyzed water solution.

Wine code	**Free chlorine addition to grape must**	**[TCA]**	**[TeCA]**	**[PeCA]**	**[TCP]**
-	*mg/L must*	*ng/L wine*	*ng/L wine*	*ng/L wine*	*ng/L wine*
M^{0}	0 (control, no addition)	nd	nd	nd	nd
$M^{+0.0005}$	0.0005	nd – 0.8	nd	nd – 28.6	nd – 8.3
$M^{+0.001}$	0.001	nd – 0.7	nd	nd – 7.0	nd – 3.7
$M^{+0.005}$	0.005	0.5 – 18.8	nd	nd – 9.3	4.7 – 60.0
$M^{+0.01}$	0.01	nd	nd – 0.5	nd – 15.3	nd – 4.9
$M^{+0.05}$	0.05	nd	nd – 1.0	nd – 25.0	5.5 – 7.0
$M^{+0.1}$	0.1	nd	nd	nd – 32.5	nd
M^{+1}	1	nd	nd	nd – 11.2	15.0 – 32.7
M^{+10}	10	nd	nd	nd – 4.2	14.0 – 22.8

M^{0} = control wine. $M^{+concentration}$ = must added with a certain concentration of free chlorine. nd = not detected. TCA: 2,4,6-trichloroanisole; TeCA: 2,3,4,6-tetrachloroanisole; PeCA: pentachloroanisole; TCP: 2,4,6-trichlorophenol.

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Table 3. Effect of 24-hour grape storage in the dark at different temperatures after electrolyzed water solution treatment on the production of chloroanisoles and chlorophenols in wines after four months from the end of alcoholic fermentation.

Wine code	**Grape storage temperature**	**[TCA]**	**[TeCA]**	**[PeCA]**	**[TCP]**
-	-	*ng/L wine*	*ng/L wine*	*ng/L wine*	*ng/L wine*
CW	Control (no EW treatment, no storage)	nd a	nd a	nd a	nd a
W^{0h}	Treated with EW and then rinsed with water (0h)	2.6 ± 1.1 ab	nd a	13.4 ± 6.4 a	62.4 ± 3.7 a
$W^{10°C}$	10 °C	5.6 ± 0.2 ab	nd a	5.3 ± 7.4 a	82.1 ± 86.1 a
$W^{20°C}$	20 °C	7.3 ± 2.7 b	0.5 ± 0.7 a	7.4 ± 10.5 a	76.3 ± 87.3 a
$W^{30°C}$	30 °C	6.0 ± 2.1 ab	nd a	4.5 ± 6.4 a	14.5 ± 7.8 a
-	*Sign.*	*	ns	ns	ns

All data are expressed as average value ± standard deviation (n = 2). Non-detects were treated as LOD/2. Different Latin letters within the same column indicate significant differences among treatments according to Tukey-b test ($p < 0.05$). Sign.: * and ns indicate significance at $p < 0.05$ and not significant, respectively, among treatments. CW= control wine. W^{0h} = wine from grapes treated with EW and immediately rinsed with deionized water. $W^{temperature}$= wine from grapes treated with EW and then stored at a certain temperature. EW= electrolyzed water. nd = not detected. TCA: 2,4,6-trichloroanisole; TeCA: 2,3,4,6-tetrachloroanisole; PeCA: pentachloroanisole; TCP: 2,4,6-trichlorophenol.

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Table 4. Effect of short-term grape storage and grape withering time in the sunlight after electrolyzed water solution treatment on the production of chloroanisoles and chlorophenols in wines after four months from the end of alcoholic fermentation.

Wine code	**Grape storage time**	**[TCA]**	**[PeCA]**	**[TCP]**
-	-	*ng/L wine*	*ng/L wine*	*ng/L wine*
Short-term grape storage				
CW	Control (no EW treatment, no storage)	nd a	nd a	nd a
W^{0h}	Treated with EW and then rinsed with water (0h)	2.6 ± 1.1 b	13.4 ± 6.4 a	62.4 ± 3.7 b
W^{4h}	4 hours	3.7 ± 0.2 b	12.3 ± 6.6 a	76.0 ± 19.8 b
W^{24h}	24 hours	2.2 ± 0.1 ab	4.9 ± 1.0 a	70.0 ± 5.7 b
-	*Sign.*	*	ns	**
Grape withering				
CW	Control (no EW treatment, no storage)	nd a	nd a	nd a
W^{96h}	96 hours	1.6 ± 0.4 ab	7.4 ± 2.8 a	67.5 ± 31.8 a
W^{168h}	168 hours	1.0 ± 0.6 ab	9.1 ± 3.5 a	61.5 ± 16.3 a
W^{500h}	500 hours	2.3 ± 0.5 b	16.5 ± 3.5 a	38.0 ± 11.3 a
	Sign.	*	ns	ns

All data are expressed as average value ± standard deviation (n = 2). Non-detects were treated as LOD/2. Different Latin letters within the same column indicate significant differences among treatments according to Tukey-b test ($p < 0.05$). Sign.: *, **, and ns indicate significance at $p < 0.05$, 0.01, and not significant, respectively, among treatments. CW= control wine. W^{0h} = wine from grapes treated with EW and immediately rinsed with deionized water. W^{time}= wine from grapes treated with EW and then stored during a certain time. EW= electrolyzed water. nd = not detected. TCA: 2,4,6-trichloroanisole; PeCA: pentachloroanisole; TCP: 2,4,6-trichlorophenol. TeCA (2,3,4,6-tetrachloroanisole) was not detected.

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Highlights

- The risk of postharvest electrolyzed water grape treatments is TCA presence in wine
- Added free chlorine in grape must resulted in TCA presence after fermentation
- Chloroanisoles and chlorophenols were also assessed in wines after grape storage
- Grape storage in sunlight significantly reduced chloroanisoles contents in wine
- Withering of treated grapes was not able to avoid chloroanisoles formation

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