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Measurement of the Effect of Pulsed Electric Fields on the Inactivation of Wine Yeasts

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***Abstract* – Traditional sterilization techniques for wine (more in general liquid food) often compromise the organoleptic properties of the food itself. Research is underway to find alternative solutions. The pulsed electric field has proved to be an alternative technique for food sterilization. However, scientific papers on this topic report conflicting results regarding treatment details such as the duration, amplitude, frequency and number of required field pulses. In this article, some preliminary experiments will be presented in the application of pulsed electric fields in winemaking, taking care of the aspects related to the measurement of the electrical quantities involved. The experiment was carried out in the first place to evaluate the selectivity of the treatment with respect to the different yeast species naturally present in grape juice. The measurement of the electrical quantities involved is not trivial, the article represents a first effort to develop a correct measurement setup, aimed at providing the scientific community with the first experimental measurement results during the treatment with the pulsed electric field.**

Keywords – Pulsed Electric Field, Pulse Measurement, Electroporation, Food Sterilization, Microbial Control in Winemaking.

I. INTRODUCTION

The safety of foodstuffs is an indispensable requirement that is taken for granted by consumers; nevertheless, nutritional quality is affected by the presence of microorganisms. For these reasons, microorganisms are one of the main concerns for the food industry. As foodborne illnesses caused by pathogenic microorganisms represent an enormous public health concern, preservation technologies are essential in order to guarantee food safety. Due to its simplicity and effectiveness, heating is one of the most frequently used methods of microbial inactivation in the food industry. Nevertheless, as heat treatments have repercussions on the nutritional and sensory characteristics of food, the interest in developing gentler methods of food preservation has greatly increased over the last decades. Pulsed electric field (PEF) is one of the most promising non-thermal technologies. It consists in applying high-voltage electric fields (5-50 kV/cm) in short pulses (µs-ms) to a matrix located between two electrodes. PEF treatments have been shown to cause microbial inactivation of bacteria, yeasts and molds; thus, this technology could be an alternative to thermal treatments.

This document deals with the possible application of PEF treatments as a microbiological control technique in winemaking. In traditional winemaking the grape juice, after being pressed, is put into vats where fermentation takes place, spontaneously transforming the juice or must into wine, without any human intervention. The principal yeast involved in wine fermentation, *Saccharomyces cerevisiae*, is not predominant in grape microbiota, which consists mostly of apiculate yeasts (genera *Kloekera* and *Hanseniaspora*) [1-2]. The presence and participation in the alcoholic fermentation of these non-*Saccharomyces* yeasts can sometimes compromise the quality of the wine causing excessive production of unwanted compounds (i.e. acetic acid, sulfur compounds) and an incomplete fermentation of sugars. For the control of unwanted microflora present in grape juice, the most common practice applied in wineries is the addition of sulfur dioxide (SO_2), due to its effectiveness in inhibiting the growth of *non-Saccharomyces* yeasts and selectively promoting the growth and dominance of *S. cerevisiae* added as a fermentation starter. However, if added in excess, sulfur dioxide may penalize the organoleptic quality of the wine and cause adverse effects on human health. Therefore, in order to prevent adverse health effects and to address WHO (World Health Organization) recommendations, the general trend in winemaking is to reduce the SO_2 content or even eliminate it altogether. Consequently, the wine industry is interested in finding alternative strategies to reduce or eliminate SO_2 in wine production, while maintaining wine quality. Among such innovative technologies, the aforementioned PEF technology is extremely promising [3–5].

At this early stage, two yeast strains, belong *S.*

cerevisiae and *H. uvarum* species, are tested separately in order to assess microbial resistance, and preliminary results are presented. As not all yeasts must be inactivated, this could allow us in the future to adjust PEF treatments, to selectively destroy only a part of the microbiota. At the same time, the electrical quantities used for the PEF treatments have been measured, as a first effort to fill the gap in the scientific literature on the subject.

II. PULSED ELECTRIC FIELD AND ELECTROPORATION

The state-of-the-art consensus is that electroporation can be best described as the formation of aqueous pores in the lipid bilayer. According to this theory, the formation of aqueous pores is initiated by water molecules penetrating the membrane's lipid bilayer. This promotes reorientation of the adjacent lipids and their polar head groups, which, being hydrophilic, begin pointing toward these penetrating water molecules. Spontaneous water penetration through the lipid bilayer can occur even in the absence of an external electric field, but these events are rare because exposure of the membrane to an electric field builds up an induced transmembrane voltage (positive and negative charge carries accumulating on either side of the membrane) and reduces enormously the energy barrier experienced by water molecules. For transmembrane voltages of several hundred mV, pores become sufficiently numerous and long-lasting to produce a detectable increase in membrane permeability.

Applying an electric field to a cell can have, in general, three possible outcomes. The outcome depends on the local field strength, the duration of the cell's exposure, and the membrane recovery rate. If the field strength and exposure time are insufficient, the cell's permeability is left unaffected. If the field strength exceeds the so-called reversible threshold and exposure lasts for an adequate amount of time, reversible electroporation occurs. It means that, as the term "reversible" implies, the membrane eventually returns to its original state when the external electric field is removed. Reversible electroporation is useful when the aim is to insert some molecules into a cell or, in contrast, to remove them from a cell.

In case field strength is too high, irreversible electroporation, which we are interested in, occurs. Irreversible electroporation results in loss of cell homeostasis, effectively killing the cell. The required amplitude level of the electric field for irreversible electroporation to occur is called *critical field*. The *critical field* threshold varies depending on the type of cell treated and, for wine yeasts, according to scientific literature, it should have a value of about 20 kV/cm (E_{crit}~$20\ kV/cm$).

The treatment must last a relatively short time (in the order of minutes or even less), otherwise, it would become impossible to treat a large amount of food, but above all, because the yeasts within the fluid reproduce rapidly in the presence of sugars (10 times in 20 minutes). Since the number of pulses is in the hundreds, the frequency must be in the order of some hertz.

Furthermore, given the presence of the electric field, an electric current flow inside the fluid; this current heat the fluid due to the Joule effect. But this is an unwanted effect because heating can alter the organoleptic properties of the wine. To avoid the Joule effect, the duration of the pulses must be as short as possible, to reduce the thermal energy transmitted to the fluid as much as possible.

Most works about electroporation have been conducted without paying the necessary attention to the electric aspects. Maybe because measurement issues, related to PEF treatments, are pretty challenging to be solved. The signals involved (i.e. voltage and current) have high levels (thousands or tens of thousands of volt and hundreds of ampere) and very short durations, with a very high slew rate (tens of GV/s), which entails high-frequency content (in the order of GHz).

These issues have been weakly addressed in the literature, and this is one of the reasons why many papers present contrasting results. However, measuring them properly is crucial, to ensure the repeatability of experiments.

III. PULSE GENERATION AND MEASUREMENT SETUP

To implement a PEF treatment, a high-voltage pulse generator is needed [6]. A laboratory system has been set up, consisting of an Alternating Current (AC) supply that feeds a Villard cascade voltage multiplier (it is a network of capacitors and diodes). A block scheme of the realized system is shown in Fig. 1.

The AC voltage has a frequency of 1 kHz and amplitude adjustable up to 300 V RMS (Root Mean Square). The Villard multiplier is composed of 24 diode-capacitor cells, thus it rectifies the voltage and introduces a scale-up factor equal to 24. The realized system is able to reach a DC (Direct Current) voltage of 10 kV. An adjustable spark gap is connected to the output of the multiplier. Varying the distance between electrodes of the spark gap, it is possible to adjust the voltage level of the generated pulses. The electric pulse generated is connected to a commercially available cuvette stand designed for reversible electroporation. The stand has been opportunely modified to withstand higher voltage levels, needed for irreversible electroporation. The generated signals have a slew rate of 20 GV/s and last as little as 2 µs. For this reason, in order to measure the pulses, instrumentation that operates in a wide frequency range is needed.

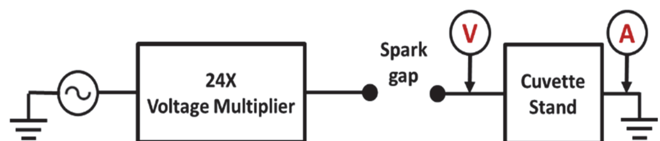

Fig. 1. Block scheme of the pulse generation circuit.

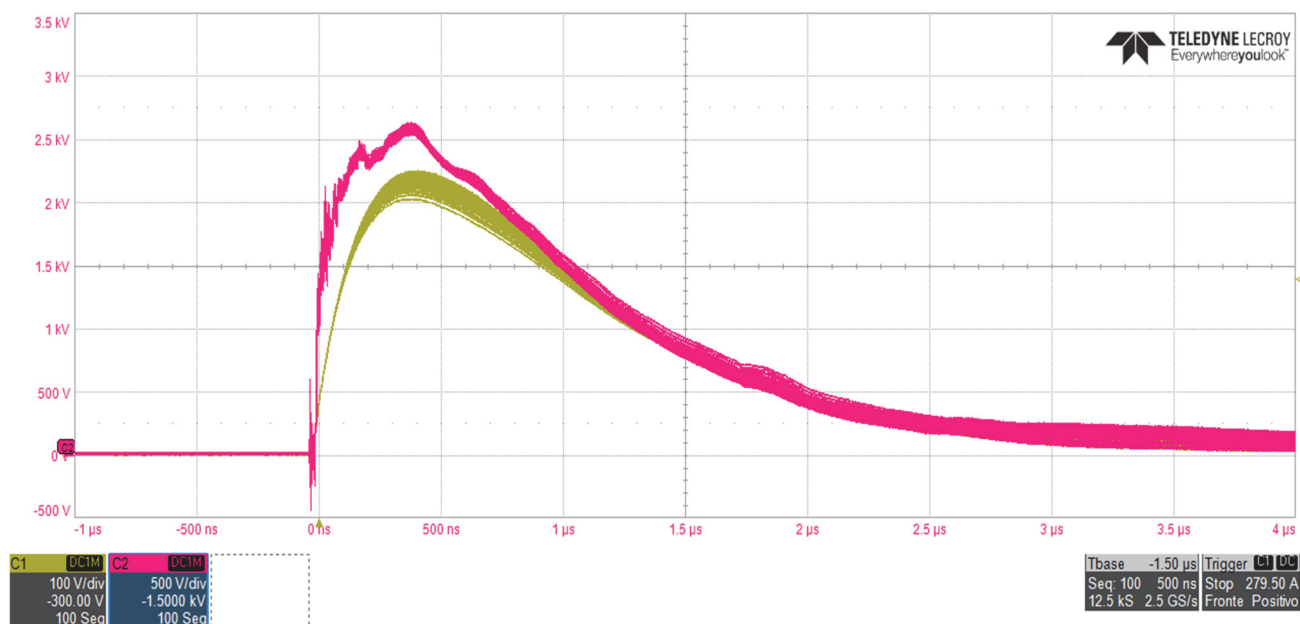

Fig. 2. Voltage and current signals waveforms measured during 100 pulses.

As a consequence, a high-performance digital oscilloscope has been adopted. In particular, the HD4096 from Lecroy, with 12-bit ADCs and a bandwidth up to 1 GHz. For the voltage channel, a high voltage probe PVM-4 from North star has been employed, it withstands up to 60 kV and has a rise time of 2.5 ns. For the current channel high accuracy current transformer, model 411 from Pearson, has been used, it has a rise time of 20 ns.

The electric field acting on the organic material inside the cuvette depends on the geometric properties of the cuvette itself, in particular on the distance between the internal electrodes (cuvette gap). In this research, cuvettes with a gap of 1 mm have been used. Each applied volt gives an electric field of 10 V/cm. To overcome the yeast critic field of about 20 kV/cm, it was sufficient to generate voltage pulses greater than 2 kV.

In Fig.2, the voltage across the cuvette stand and current flowing during a pulse are depicted. The oscilloscope was configured in sequence mode. This working mode allows the acquisition of multiple waveforms with the same trigger configuration. This has been used to evaluate the performance of the generation systems in terms of repeatability. In Fig.2, the acquisition of 100 pulses is reported. As it can be seen, the pulses overlapped quite well. The vertical scale is 500 V/div for the voltage channel and 100 A/div for the current one. It can be noted that the voltage pulse has a peak slightly over 2.5 kV and the current during the pulse overcomes 400 A. This entails a peak power greater than 1 MW.

To evaluate the impact of temperature on yeast inactivation, the temperature inside cuvettes was monitored during the tests by adopting a thermal camera. The temperature rise was negligible, for this reason also the effect on yeast can be neglected.

IV. DESCRIPTION OF THE TESTS

The *S. cerevisiae* AM3C and *H. uvarum* CM12V strains belong to the DiAAA (University of Molise, Campobasso Italy) collection and the respective DNA sequences have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers: OM037665 and OM038322.

Single pure cultures were obtained after aerobic growth at 25 °C in YPD (1% w / v yeast extract, 2% w / v peptone and 2% w / v dextrose). After 12 hours the cultures were centrifugated (7000 x *g*) and the harvested cells were inoculated into PBS pH 7.4 (phosphate buffered saline: 37 mM Sodium chloride, 2.7 mM Potassium chloride, 10 mM Disodium hydrogen phosphate, 1.8 mM Potassium dihydrogen phosphate) to obtain an initial live cell density of about 10^5 and 10^7 CFU/mL (Colony-Forming Unit). Single-cell suspensions of *S. cerevisiae* AM3C and *H. uvarum* CM12V in PBS not subjected to any treatment have been used as controls.

Therefore, the individual yeast cultures underwent the survival tests reported in Table 1:

Table 1. Survival tests.

	CFU/mL	n. pulses
Test A	10^7	100
Test B	10^7	400
Test C	10^5	100
Test D	10^5	400

Before and after each treatment viable counts of the surviving yeasts were determined by standard plating technique. Every sample was serially diluted in sterile 0.9 % saline solution (dilution 1:10), plated in Petri dishes

containing WL nutrient medium and incubated at 30 °C for 48 hours before counting. All chemical compounds and microbial media were purchased from Sigma-Aldrich (St. Louis, MO, USA). All experiments were performed in triplicate and the data are expressed as the mean ±standard deviation.

V. EXPERIMENTAL RESULTS

In tests with 100 pulse frequencies and an initial cell density of 10^7 CFU/mL, the microbial counts showed a decrease in viability of 1 logarithmic cycle for *S. cerevisiae* AM3C and 2 logarithmic cycles for *H. uvarum* CM12V after PEF applications. The microbial counts after 2 hours from the treatment confirmed these data excluding reversibility of the electroporation phenomena in this timeframe.

In tests with 400 pulse frequencies, a greater inactivation of yeasts was obtained, and in particular, the cellular viability of *S. cerevisiae* AM3C decreased by 2

Table 2. Cell survival of S. cerevisiae and H. uvarum in PBS after PEF treatment at 2kV with 100 and 400 pulse frequencies. Results are shown as mean ± standard deviation (n = 3).

Test	Vital cell density (log CFU/mL)	
	S. cerevisiae AM3C	*H. uvarum* CM12V
Control	7.28 ± 0.06	7.19 ± 0.06
A: 100 pulses	6.47 ± 0.04	5.65 ± 0.09
B: 400 pulses	5.41 ± 0.07	< 10

Test	Vital cell density (log CFU/mL)	
	S. cerevisiae AM3C	*H. uvarum* CM12V
Control	5.12 ± 0.02	5.09 ± 0.02
C: 100 pulses	< 10	< 10
D: 400 pulses	< 10	< 10

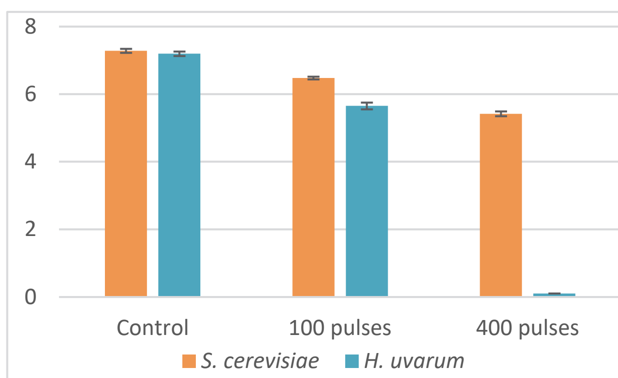

Fig. 3. Survival of S. cerevisiae AM3C and H. uvarum CM12V in PBS with an initial cell density of 10^7 CFU/mL after PEF.

logarithmic cycles whereas a complete inactivation of *H. uvarum* CM12V was obtained.

In tests in which the initial cell viability, was 10^5 CFU/mL there was complete effectiveness of the treatments with 100 and 400 pulses that caused the complete inactivation of the yeasts confirmed even in the microbial counts carried out after 2 hours (data not shown).

VI. CONCLUSIONS

The results showed a different response to the treatments of *S. cerevisiae* AM3C and *H. uvarum* CM12V. Therefore, although preliminary, the data showed that PEF could be applied in the pre-fermentation phase to inhibit the native microflora of the grapes and, in particular, the non-*Saccharomyces* yeasts by promoting the predominance of *Saccharomyces* yeasts indigenous or added as starters in the must be fermented. However, further tests must be carried out in the future using grape must and wine to evaluate the efficacy and the contribution that the application of PEF can make as an alternative technique to the use of SO_2 in winemaking. Further experiments at different voltage levels and/or changing frequency, duration and number of pulses, must be conducted, to assess the optimal parameters of the PEF treatment needed for the predominance of *Saccharomyces* yeasts.

REFERENCES

[1] **Lombardi SJ, Pannella G, Iorizzo M, Moreno-Arribas MV, Tremonte P, Succi M, Sorrentino E, Macciola V, Di Renzo M, Coppola R**. Sequential inoculum of *Hanseniaspora guilliermondii* and *Saccharomyces cerevisiae* for winemaking Campanino on an industrial scale. World J Microbiol Biotechnol. 2018 Oct 24;34(11):161.

[2] **Testa, B., Lombardi, S.J., Iorizzo, M. et al.** Use of strain Hanseniaspora guilliermondii BF1 for winemaking process of white grapes Vitis vinifera cv Fiano. Eur Food Res Technol 246, 549–561 (2020).

[3] **C. Delsart, N. Grimi, N. Boussetta, C. M. Sertier, R. Ghidossi, M. M. Peuchot, and E. Vorobiev**. Comparison of the effect of pulsed electric field or high voltage electrical discharge for the control of sweet white must fermentation process with the conventional addition of sulfur dioxide. Food Research International 77, 718–724 (2015).

[4] **Ozturk, Burcu; Anli, Ertan; Aurand, Jean-Marie** (2017). *Pulsed electric fields (PEF) applications on wine production: A review. BIO Web of Conferences, 9(), 02008–* . doi:10.1051/bioconf/20170902008.

[5] **Silva FVM, van Wyk S. Emerging** Non-Thermal Technologies as Alternative to SO_2 for the Production of Wine. Foods. 2021 Sep 14;10(9):2175.

[6] **M. A. Elgenedy, A. M. Massoud, S. Ahmed, B. W. Williams and J. R. McDonald**, "A Modular Multilevel Voltage-Boosting Marx Pulse-Waveform Generator for Electroporation Applications," in IEEE Transactions on Power Electronics, vol. 34, no. 11, pp. 10575-10589, Nov. 2019, doi: 10.1109/TPEL.2019.2899974.