STUDY OF THE EFFECT OF HIGH INTENSITY PULSED ELECTRIC FIELD TREATMENT ON NATURAL MICROBIAL POPULATION OF MUSTS

PUIG, A.* [(1)], MARSELLÉS, R. [(2)], OLMOS, P. [(1)], MARTÍN, O. [(2)] and MÍNGUEZ, S.[(1)]
[(1)] Institut Català de la Vinya i el Vi (INCAVI)-IRTA. C/ Amàlia Soler, 29. 08720 Vilafranca del Penedès (Barcelona) (Spain). Telf .+0034 93 890 02 11. Fax. +0034 93 890 03 54; e-mail: apuigpujol@gencat.net
[(2)] Departamento de Tecnología de los Alimentos. UTPV-CeRTA. Universidad de Lleida. Avda. Alcalde Rovira Roure, 191. 25198 Lleida (Spain).

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INTRODUCTION

The elaborator sector of musts, wines and derivatives of the grape needs to guarantee the microbiological and biochemical stability of finished products, with the intention to ensure commercial acceptance. On the other hand, this sector, forced to respect the natural product character of the wine, sees restricted additives used to maintain this stability. The use of these additives has two types of problematic issues: one related with food safety, as the case of the progressive diminution of the sulphur dioxide content in the elaboration because of the possible risk of allergic reactions in some individuals, and another related with technological aspects, as it is the case of certain organic acids: ascorbic acid and sorbic acid that can produce sensory deviations.

Recently, it has been proposed and approved the use of other additives: lysozime, dimethyldicarbonate, etc. Nevertheless, after being approved by different sectorial institutions (O.I.V., states, etc), they are also limited by a specific legislation for the wine sector. In spite of this, the previous products and other alternative products that are still in the experimental phase, are seen with precaution by the cellars, given that they are very careful to keep the wine natural conditions. This caution is not so accused with the use of physical techniques: heat treatments, filtration, high pressures, etc. With the purpose of extending the possibilities and resources of the oenologist, we have studied the microbiological effects of certain physical processes that in principle do not have repercussions in the final sensory quality. This is the case of high intensity pulsed electric field (HIPEF) (Barbosa-Cánovas, G.V. et al., 1999).

The application of HIPEF is a nonthermal technology that is being applied to avoid the negative incidences that heat produces on foods. Due to their electrical characteristics, fruit juices were the first foods where HIPEF were applied (Qin, B.L. et al., 1995), obtaining elevated inactivations of microorganisms, the main target of any stabilization and conservation technique. The studies on the microbial behaviour under treatment by HIPEF have been made mainly in peach (Arántegui, J. et al., 1999), apple (García, D. et al., 2005) and orange juices (Elez-Martinez, P. et al., 2005). These works have demonstrated that microorganisms are destroyed in greater or smaller degree depending on the type of juice, on the type of microorganism, the equipment used and electrical parameters applied. Microbial behaviour under HIPEF treatments in grape juice or must has received little attention. There are only two reports about the effects of HIPEF treatments on spontaneous spoilage microorganisms (total yeast and bacteria) occurring in grape juice (Jaya, S. et al., 2004; Wu, Y. et al., 2005) but there are no studies in specific species. INCAVI and the Department of Food Technology of the University of Lleida considered to evaluate the possibilities that this technique offers to reduce the microbial load of musts, determining the more suitable conditions of treatment and its repercussions on sensory and oenological parameters. The treatment by HIPEF was compared with a traditional treatment of juice conservation: the pasteurization.

MATERIALS AND METHODS

Samples: preparation and microbiological analysis

In order to determine the most microbiological inactivation by HIPEF method, pasteurized Parellada variety must was used. Pasteurized musts were inoculated with five stocks of yeast and representative bacteria of the natural flora that can be found in a must. Microbes inoculated were *Saccharomyces cerevisiae* strain P29, which was obtained from the yeast wine collection of the Instituto Catalán de la Viña y el Vino (INCAVI) (Vilafranca del Penedès, Barcelona, Spain), and *Kloeckera apiculata* (*Hanseniaspora uvarum*) CECT 11105 (Universidad de Valencia, Valencia, Spain), as typical representatives of yeast whereas *Lactobacillus plantarum* strain C11 (INCAVI), *Lactobacillus hilgardii* CECT 4786 (Universidad de Valencia) and *Gluconobacter oxydans* LMG 1408 (Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium) were taken as usual lactic and acetic bacteria present in grape juice.

Cultures of yeast and bacteria were allowed to grow separately in grape juice at 30ºC in three successive steps of propagation in order to become acclimatized in this medium until reaching 100 ml of culture at the end of exponential or beginning of stationary phase. The combination of the five cultures yielded 500 ml that were added to 500 ml of fresh grape juice. The final 1 L volume contained 10^6-10^7 CFU · ml^{-1} of each microorganism. To evaluate the viable cells, grape juice samples were serially diluted with the appropriate Ringer buffer solution when necessary, filtered through 0.45 µm pore diameter nitrocellulose sterile filters (Whatman International Ltd, Maidstone, England) and plated on selective media and growing conditions. *S. cerevisiae*: YEP medium (yeast extract 5%; glucose 2%; peptone 2%; agar-agar 2,7%) supplied with chloramphenicol (500 mg/L) and ethanol 13% after sterilization. Plates were incubated at 30ºC in a heater with 5% CO_2 in the atmosphere for 7 days. *K. apiculata*: YEP medium supplied with chloramphenicol (500 mg/L) and cycloheximide (50 mg/L) after sterilization.

Plates were incubated at the same conditions as *S. cerevisiae* for 5 days. Lactic bacteria (*L. plantarum* and *L. hilgardii*): *Oenococcus oeni* medium (MLO) with 2% agar-agar and supplied with nystatin (50 mg/L) after sterilization. Growing conditions were at 30ºC into an anaerobic jar in a heater with 5% CO_2 in the atmosphere for 6 days. *G. oxydans*: GY medium (glucose 5%; yeast extract 1%; agar-agar 2%) supplied with nystatin (50 mg/L) and penicillin G (3 U/ml) after sterilization in an aerobic atmosphere at 28ºC for 7 days. Chloramphenicol, cycloheximide, nystatin and penicillin G were purchased from Sigma-Aldrich Inc (Steinheim, Germany). Glucose, soy peptone and ethanol were provided by Panreac Química S.A. (Barcelona, Spain). MLO, yeast extract and agar-agar were supplied by Scharlau Chemie S.A. (Barcelona, Spain) Each one of the tried treatments was made by duplicate in the same must and two separated must batches in the time (n=4).

The obtained results were interpreted as the degree of destruction or microbial inactivation calculated as the logarithm of the relation between the number of viable cells of each microorganism of the must sample without treatment (No) with respect to the same sample after the treatment (N): (log (No/N)). The same experimental conditions were repeated with the treatment that better had worked, on other musts obtained in the experimental cellar of INCAVI. The other musts varieties were: Moscatell de Alexandria (chosen like aromatic white variety), Mazuelo (red variety) and Parellada (neutral aromatic white variety). In these musts, not only microbiological effects were studied, but also oenological and sensory characteristics, comparing the same fresh must batch without receiving treatment (control), treated by HIPEF (HIPEF) and pasteurization treatment (pasteurized) in the conditions that are detailed in the following section.

HIPEF equipment and treatments

The used equipment is showed in Figure 1. The electrical part consist of an energy charging unit OSU-4F (Ohio State University, Columbus, OH, USA), an electronic trigger 9412A (Cuantum Composers, Inc., Bozeman, MT, USA), eight treatment chambers made of stainless steal and

Delrin® arranged in series and, an oscilloscope THS720 (Tektronix Inc., Beaverton, OR, USA). The pulse generator module can deliver square wave pulses of 1-12 kV and 1-10 µs with the same or alternate polarity at a rate up to 2000 Hz. Grape juice flowed through the treatment chambers and received electric field pulses each time it crossed between the electrodes, which were separated each other 0.29 cm.

Figure 1. HIPEF equipment OSU 4F used in this study for must treatment.

The system is completed with a gear pump (Cole Palmer Inc., Vernon Hills, IL, USA), a tubing system with refrigerating coils, which are made of food grade stainless steal, temperature probes attached to the inlets and outlets of the treatment chambers, and a heat exchanger. The maximum flow rate of grape juice was 3.33 $ml \cdot s^{-1}$ to keep under control the temperature of the product. Therefore, the maximum value reached by any temperature probe at the outlet of the treatment chambers was 30ºC whereas the inlet was approximately 15ºC. Temperatures as well as all electrical data were controlled and gathered in a computer. In order to determine the optimal conditions of HIPEF treatment to reduce to the maximum the microbial load of musts, different parameters combinations were tested. A bipolar square waved pulses of 5 µs were fixed, the intensity of electric field strength (E) varied between 20, 25, 27.5, 30 and 35 kV/cm, pulse frequency (F) of 50, 100 and 300 Hz and treatment time (t) of 0.125, 0.250, 0.500, 0.750 and 1.000 ms.

Pasteurization of musts of the varieties Moscatell, Mazuelo and Parellada was made by means of an equipment of pasteurization of scale laboratory and tubular type. The equipment consists of a peristaltic pump and two water baths at different temperatures in which a stainless steel pipe allows to maintain the product to the wished temperature. In the pasteurization stage the temperature of the bath stayed at 90ºC and the volume of the pump adjusted to obtain 1 minute of total time of processing after which the must was cooled quickly until 5ºC by means of another bath at 2ºC.

Oenological and sensory analysis

In order to study the possible repercussions that HIPEF can exert on musts, compared with a classic treatment by pasteurization and the same must without receiving any type of processing, the following analysis were made: oenological parameters: specific gravidity, titrable acidity, pH, ºBrix and soluble solids (D.O.C.E., 1990), phenolic compounds: total polyphenols, non-flavonoid compounds (Kramling, T.E. and Singleton, V.L., 1969), catechins (Vivas, N. et al., 1994) and hidroxicinnamate acids or A_{320} (Zoecklein, B.W., et al., 1995), chromatic parameters: A_{280}, A_{420}, A_{520}, A_{620}, hue, intensity (Glories, Y., 1984) and CIELAB parameters (Bakker, J. et al., 1986).

A study at sensory level was made to know what thought a group of people (simulating possible consumers) with respect to the visual aspect, aroma and taste of musts treated by HIPEF or pasteurized and the same must without treatment. With this purpose, two types of tasting were made: a preference tasting, hedonistic type, in which it was asked to non-expert people (a minimum of 20 individuals, making the same tasting by duplicate) about the three batches of the must (HIPEF, pasteurized and control) valued in a scale from 1 ("I do not like") to 9 ("I like very much"). In addition a descriptive tasting was made by means of a panel of 8 expert tasters in which they scored in 1 to 9 scale the colour, intensity of aroma, fresh fruit aroma, cooked fruit aroma (like negative property in a fresh must), intensity of taste, fresh taste, final sensation in taste and global evaluation of the tasted must. Results were analyzed by means of an ANOVA test ($P<0.05$) in order to know if non differences between the treatments existed. In case there were differences, a Tukey test was conducted in order to determine which of the three treatments (HIPEF, pasteurization and control must) caused those differences.

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RESULTS AND DISCUSSION

Conditions of processing by means of HIPEF more apt for the destruction of must microorganisms

As in any microbiological stabilization technique, the first step was to study the microbial inactivation by means of HIPEF. This inactivation depends on factors like: the intensity of electric field, the time of treatment, the number of applied pulses and the width, the frequency and the type of applied pulse (Qin, B.L. et al., 1996). In order to observe the behaviour of the microorganisms inoculated in must, up to 75 different HIPEF processing combinations were tested by duplicate. By means of selective culture media, destruction level on each group of microorganims was determined. Obtained results allowed to elaborate inactivation graphs in which it was possible to observe the combinations of parameters that better affected the destruction of the flora that can be found in must grape. With treatments at 35 kV/cm of electric field strength, 5 μs of pulse width and 300 Hz of frequency during 1000 μs we obtained an inactivation between 3.5 and 4 log for the two species of yeasts and the lactic acid bacteria and up to 2.5 log for the acetic bacteria (Fig. 2).

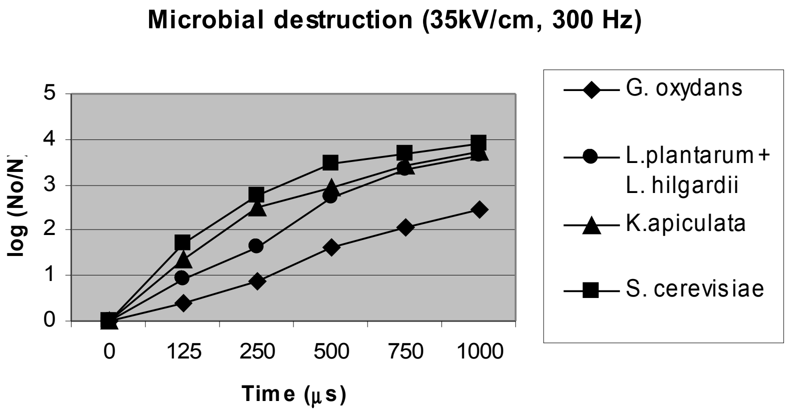


Figure 2. Microbial destruction of different strains inoculated in must treated by HIPEF at different treatment times, 35 kV/cm of electric field strength and 300 Hz of pulse frequency.

As it has been verified in other studies, the microbial reduction in grape must depends on the type of microorganism: eukaryotic cells are, in general, more sensible than prokaryotes (Pothakamury, U. R. et al., 1995), on the concentration of initial inoculum: the higher the concentration, less effective the treatment is (Zhang, Q. et al., 1994), on the growth phase of the culture: the cells in logarithmic phase are more sensitive to the HIPEF than those that are in stationary phase (Pothakamury, U.R. et al., 1996) and on the ionic concentration of the medium in which they are: the smaller ionic force of the product, the greater microbial inactivation (Martín, O. et al., 1997). For this last reason, in order to make comparisons of results, it is advisable to do it in a same type of medium.

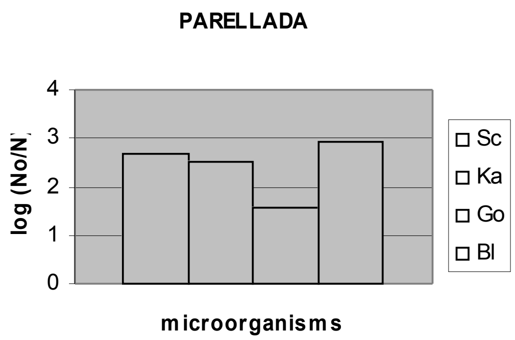
Some results have been published about the effects of HIPEF on the population of spoilage yeast of commercial grape juice (Jaya, S. et al., 2004; Wu, Y., et al, 2005). Wu et al. (2005) reported 4 and 4.2 logarithm reductions of common spoilage flora of white and red commercial grape juice. Even so, the HIPEF equipments and treatments applied were completely different and they even needed to heat grape juice up to 50ºC to reach those values. In our case, the temperature never surpassed 30ºC and, therefore, this factor did not influence on the obtained degree of microbial destruction. However, those works reach the same conclusion that has been observed in the present study: treatment time and electric field strength were the most significant factors affecting spoilage flora and the results improved as their values were increased.

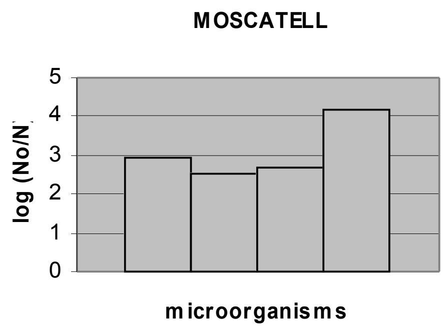
With respect to the bacterial behaviour, according to the consulted bibliography, it does not exist information about must treated by HIPEF. There are some results on orange juice about *Lactobacillus* genus, where they obtained up to 5.8 logarithmic reductions in *L. brevis* (Elez-Martínez, P. et al., 2005) and in juice of orange and carrot (Rodrigo, D. et al., 2001) where a 2.5 log reduction of *L. plantarum* was obtained when treating the juice at 35 Kv/cm. Data has not been found about the evolution of acetic bacteria as is the case of *Gluconobacter oxydans*. Like in the case of yeast, it was corroborated that the electric field strength and the time of treatment are the main factors of destruction.

Considering that the obtained inactivation took place in artificially inoculated musts and that in normal conditions the existing natural population in must does not surpass in most of the cases the inoculated levels, the technique of HIPEF seems to be a good alternative for the oenological sector.
The obtained results also allowed us to construct a mathematical model based on the response surface methodology. A second order polynomial model permits to predict and anticipate the reduction of the microorganism population in must applying certain conditions of HIPEF with no need of making new experiments (Marsellés, R. et al., personal communication).

Microbiological repercussions in natural must treated with HIPEF compared with a treatment by pasteurization

The second part of our work consisted in the study of the microbiological repercussions in three musts obtained from traditional way in cellars: Moscatell, Mazuelo and Parellada, without adding SO_2. The three musts were inoculated with the same microorganisms of the first part and processed in the most effective conditions of HIPEF determined in the previous phase. This treatment was compared with a traditional processing of microbiological stabilization of juices by means of heat (pasteurization at 90ºC during 1 min) and with fresh musts not treated at all (must control). Each one of the experiments was made by duplicate.
Results of microbial destruction obtained in the three musts treated with HIPEF with respect to control musts are shown in Figure 3. The population reduction, in the case of the yeasts (*S. cerevisiae* and *K. apiculata*), were slightly inferior with respect to that obtained in the first part of the study. Also, in the Parellada variety, the acetic bacteria and the group of lactic bacteria showed inferior inactivation, 1.58 log and 2.93 log respectively. These differences could be explained to the different ionic composition of the used musts (Martín, O. et al., 1997). Nevertheless, the treatment by pasteurization showed, in all the cases, greater microbial destruction than the treatment by HIPEF. Previous works with other fruit juices (orange, peach, apple) had confirmed this fact (Raso, J. et al., 1998).





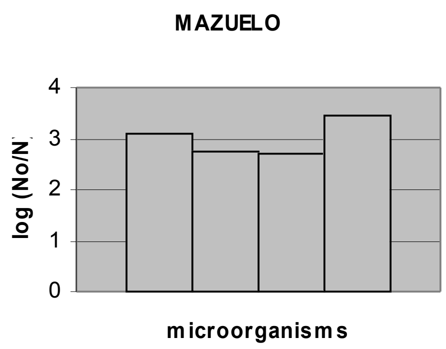


Figure 3. Levels of microbial inactivation in musts of three varieties of grape treated by HIPEF (E=35 kV/cm, F=300 Hz, t=1 ms) with respect to such musts without receiving treatment.

HIPEF effect on oenological properties of musts

Regarding oenological characterization, we did not found any differences in physicochemical parameters (specific gravidity, titratable acidity, pH, ºBrix and soluble solids) in any of the three types of analyzed musts. However, small variations in chromatic parameters and phenolic compounds were detected related to the oxidation (hue, A_{320}, A_{420}, Cielab (b), Cielab (H)) in white varieties (Parellada and Moscatell): musts control and treated by HIPEF showed a degree of oxidation (brown colour) higher than pasteurized musts. This factor can be due to a residual activity or insufficient inactivation of oxidative enzymes (polyphenol oxidase) in musts treated by HIPEF, fact that already had been described by other authors in other types of juices (Van Loey, A. et al., 2002).

Comparative sensory analysis between musts treated by HIPEF, pasteurized and control

In general, sensory evaluations that have been made in orange or apple juices treated by HIPEF indicate that the flavour of foods is not deteriorated significantly after this type of treatment (Qin, B.L. et al., 1995). However, according to our knowledge, data not exist about grape musts. In this case, sensory analysis is an important step if we take into account that later it has to transform into wine. For this reason, in this study it was decided to make two types of tasting: one with a panel of non-expert or consuming tasters (preference tasting) and another one with expert tasters in the sensory analysis of musts and wines (descriptive tasting).

The sensations of the consumers in the preference tasting varied according to the type of must. Table 1 shows the average scores obtained by each treatment and type of must and its level of significance in the analysis of the variance (ANOVA, P<0,05). In musts of the neutral variety Parellada, the treatment by HIPEF was not sensory different from pasteurized musts or control musts. However, in the aromatic variety Moscatell, significant differences between the treatment by HIPEF and must control were found, being the last one the best. With respect to the Mazuelo variety, statistical differences between HIPEF, pasteurized and control were not found although the red must treated by HIPEF was slightly better valued than the other two.

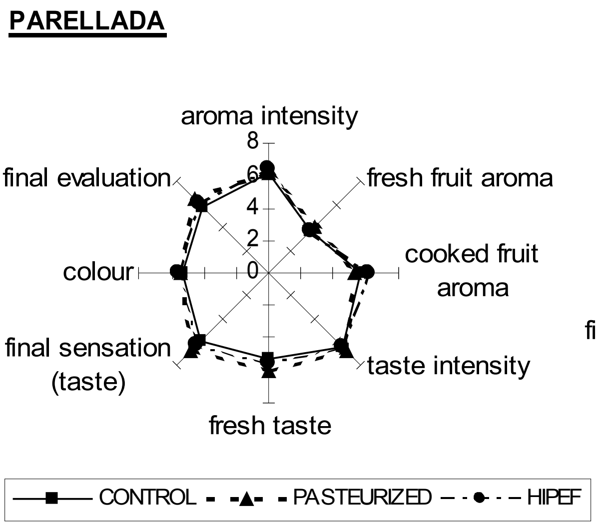
Table 1. Preference tasting: results of the analysis of variance in three musts for the three blocks of conducted treatments.

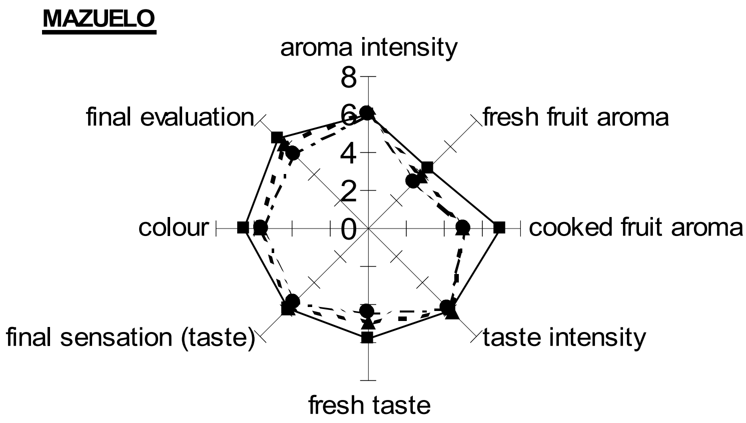
MUST	PARELLADA (n=78)			MOSCATELL (n=74)			MAZUELO (n=77)		
TREATMENT	CONTR	PAST	HIPEF	CONTR	PAST	HIPEF	CONTR	PAST	HIPEF
AVERAGE	5,80	6,03	5,84	6,35	6,07	5,86	6,01	5,90	6,17
P (ANOVA)[1]	0,175			0,014[2]			0,129		

[1] Signification level P<0.05

[2] When Tukey test (P<0.05) was applied to determine which treatment causes the differences, statistical differences between control and HIPEF group were found (P=0.01).

In descriptive tasting made by expert tasters (Figure 4), must of the neutral aromatic variety Parellada was similarly valued in the three batches: control, pasteurized and HIPEF, although the scores were slightly higher in many descriptors for the pasteurized treatment. With respect to the aromatic variety Moscatell, must control was the best one in most of the descriptors. However, in the final valuation, the treatment by HIPEF slightly was better valued. In the case of red must of Mazuelo variety, HIPEF treatment was valued as similar as pasteurized treatment and must control was slightly better scored than the previous ones, although it also received the greater score in the descriptor of negative character of cooked fruit. In general, according to the results of sensory analysis, a clear preference with respect to the type of conducted treatment did not exist and the opinions of the tasters varied according to the type of must.





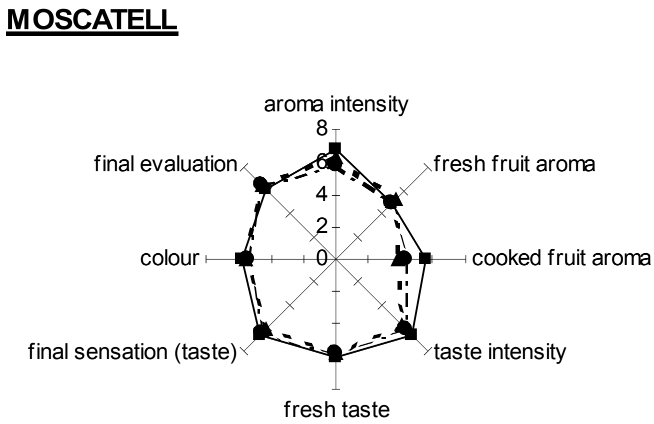


Figure 4. Scores obtained from control, pasteurized and HIPEF treatments in the descriptive tasting of three musts.

CONCLUSIONS

The must processing by means of HIPEF allows to reduce the population of *S. cerevisiae*, *K. apiculata*, *G. oxydans, L. plantarum* and *L. hilgardii,* native microorganisms in musts when entering at the cellar. Application of HIPEF in oenological industry could be introduced in the process of microbiological stabilization of must for making wine or grape juice. Its application can be an alternative to the heat treatment or the additive use like sulphur dioxide, ascorbic acid or sorbic acid, obtaining products without changes in sensory and nutritional properties. Results obtained until now with HIPEF also show that if we want to prevent the enzymatic oxidation of must, it could consider the combination of this technique with the use of sulphur dioxide or ascorbic acid, although, in smaller doses.

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