

Optical and AFM microscopy of grape juices treated with UHPH: Effects of microstructure and nanostructure

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Abstract. UHPH treatment of *Vitis vinifera* must for winemaking leads to fragmentation of colloidal particles into smaller structures. The shear and fracture forces experienced by grape juice during valve pressurization are sufficient to reduce the particle size of grape juice to below 500 nm. As a result, the applied force can disrupt bacterial and yeast cell structures, altering or breaking down proteins, polysaccharides and enzymes. This effect is not observed for low molecular weight compounds such as monomeric pigments and phenolic structures, varietal aroma precursors, fermentable sugars, etc. Treated and untreated samples can be compared using optical and atomic force microscopy. Optical microscopy images show reduction or elimination of bacteria and yeast and changes in microstructure. On the other hand, in addition to describing topography in the nanometer range, AFM can also measure particles in comparison to other techniques such as laser diffraction (LD). This work contributes to the characterization and better understanding of the effects of UHPH on grape juice for winemaking.

1 Introduction

The use of Ultra High Pressure Homogenization (UHPH) or High Hydrostatic Pressure (HHP) in grape must processing is an effective alternative to the use of sulphites to control grape plum microbes [1,2]. This technique is also a high-pressure technique, but works in continuous mode and is also used for the homogenization of other products such as yeast lees to reduce the aging time of the wine in contact with them [3]. When homogenizing with UHPH, both the colloidal particles of grape juice and the microorganisms they contain are subjected to cutting and beating forces. This technology not only completely destroys living microorganisms, but also has the ability to eliminate spores [4].

The complete removal of microorganisms using UHPH can be considered a cold pasteurization process because it does not reach the temperatures commonly used to sterilize food. At lower temperatures, it is considered a sensory protection technique, as the process does not destroy the covalent bonds of the pigments or molecules responsible for flavor development [5,6].

Homogenization using UHPH was found to reduce the size of juice particles to a size range between 100 and 300 nm [7]. These quantities are difficult to measure because sensitive techniques are required. Atomic force microscopy (AFM) and laser diffraction (LD) can

determine particle size in the nanometer range (ref), and complement other analytical techniques that help understand the intense fragmentation grape juice undergoes.

In order to better understand the effect of UHPH on the micro and nano structure of grape juices, we have carried out microscopic observations of fresh and freeze dried samples. The techniques used for these experimental trials were optical microscopy (OM) and atomic force microscopy (AFM). In the meantime, measurements done with laser diffraction (LD) were also done to determine the particle size distribution a treated juice.

2 Materials and methods

2.1 Grape musts

The musts used in this experimental set up were obtained from two wine producing regions in Northern Spain. Tempranillo must from D.O. Ribera del Duero and Verdejo juice from D.O. Rueda. The red must was obtained after 48h maceration at low temperature, and the white must was cold pressed, stabilized through cold settling and kept at 4° C until the UHPH treatment.

2.2 UHPH treatment

UHPH treatment was conducted with the equipment from Ypsicon (Ypsicon Advanced Technologies, Barcelona, Spain). All juices passed through 100 micron sieves in order to remove seeds, large size particles and crystals formed at low temperature. The conditions followed during high-pressure treatment were: flow-rate 60 L/h at 3070 ± 30 bar, inlet temperature 23-25 °C, in-valve temperature 88-90 °C for only 0.02 s and outlet temperature 20-22 °C [8]. In the case of Tempranillo must, a third sample was obtained at 2400 bar as it was observed that this must was less abrasive under this conditions.

Samples were labeled according to the diagram shown in Fig. 1. Samples labels contain the source of the juice and whether they had UHPH treatment or not.

2.3 Sample preparation

One drop of each sample was placed on circular slides, freeze dried at -80 °C and afterwards lyophilized. The samples were kept in desiccators to avoid rehydration.

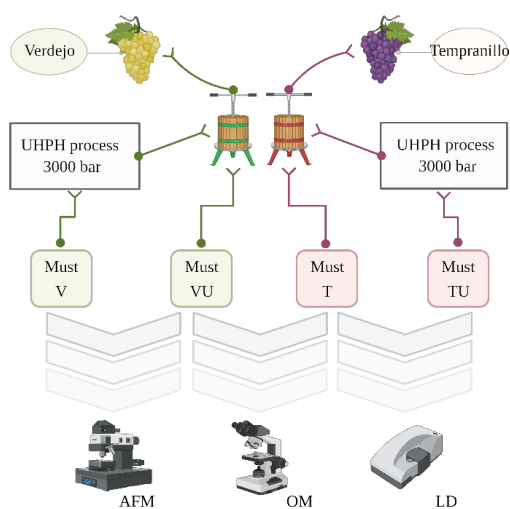


Figure 1. Experimental set up for untreated and treated grape juices. V – Verdejo, T – Tempranillo, U – UHPH.

2.4 Atomic force microscopy (AFM)

Topographic observations were done with a Nano-Observer AFM (Concept Scientific Instruments, Les ULIS, France) operating in resonant mode. A 1 N/m rectangular silicon cantilever (model Fort, AppNano, Mountain View, CA, USA) with an 8 nm nominal tip radius was selected. Amplitudes set point were 4-5 V [9].

2.5 Optical microscopy (OM)

The juices are observed with the OM in fresh and freeze dried preparation. For the observation in fresh, one drop

is placed on a slide and covered with a cover slip. The objectives used for the observation were large objective lenses 40x and 60x.

2.6 Laser diffraction (LD)

Malvern Mastersizer 2000® (Malvern Instruments Ltd., Malvern, UK) was used to determine particle size by laser diffraction. Prior to the proper laser obscuration values (5-10%), the sample was diluted with distilled water. Both the sample and the water had refractive indices adjusted at 1.340 and 1.333, respectively. The parameters d3.2, d4.3, and D50 and D90 were used to describe the particle size distribution [8].

2.7 FT – Infrared spectroscopy (FTIR)

The main composition parameters (sugar concentration, pH, concentration of tartaric acid and yeast assimilable nitrogen) were measured by FTIR analysis (OenoFossTR, Foss Iberia, Barcelona, Spain). The samples were stirred using a vortex mixer to remove entrained air bubbles to avoid interference. 1 mL of must is required for the analysis.

3 Results and Discussion

The optical microscopy images for treated and untreated samples (Fig. 2) show differences attributed to the size of particle in each case. Pigmented vegetal tissue observed in both musts without any UHPH treatment are an evidence of larger structures present in the juices before the pressure homogenization. These structures are purple in Tempranillo must (Fig. 2.a), while they appear green in the Verdejo one (Fig. 2.c).

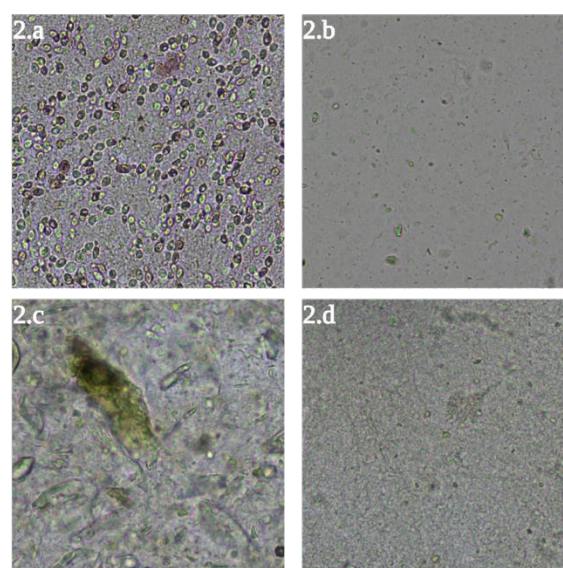


Figure 2. Observations under optical microscope for (2.a) untreated Tempranillo must and (2.b) after UHPH treatment; (2.c) untreated Verdejo must and (2.d) after UHPH treatment.

The structure of the untreated juice samples is in general rougher and, in some cases, yeast cells are also visible. Such is the case of Tempranillo must which had a large native yeast population (Fig. 2.a) as this must lacked the stabilization at low temperature obtained in the Verdejo juice.

Another observation is the loss of structure after UHPH treatment in both cases, as they seem less defined and more homogeneous afterwards. There is a reduction/elimination of cellular structures (Figs. 2b and d) as UHPH homogenization is able to produce cell disruption of microbial populations found in juices [10], and a reduction of particle size of dispersed liquid systems [3]. Therefore, the possibility of finding these biological structures is reduced to almost zero. Bright spots found in treated juices belong to crystalline structures potentially formed right after the change in pressure at the exit of the valve. From an optical point of view, there were no differences observed in Tempranillo juices treated with either 2400 bar or 3070 bar (OM images for 2400 bar treatment are not shown).

Nonetheless, and despite the fact that the structure has been modified at micro scale, it is impossible to assess the changes produced by the UHPH treatment with this technique in terms of particle size modification.

The images obtained with atomic force microscopy showed differences at working areas of 100 and 50 μm . The first was used for untreated musts, whilst the latter was selected to observe particles in UHPH treated musts. In the case of Tempranillo musts (Fig. 3), the observation was done in all three samples, untreated, treated with 2400 bar and treated with 3000 bar. The images shown for Verdejo musts (Fig. 4) include observations of untreated must and must treated with 3000 bar.

The term homogenization in this technology refers to the particle size decrease by modifying the structure of larger molecules present in a dispersed liquid system [3]. The effect observed in the ultra-high process at pressures near or above 300 MPa is observed in these two images (Figs. 3 and 4) for Tempranillo and Verdejo fresh musts.

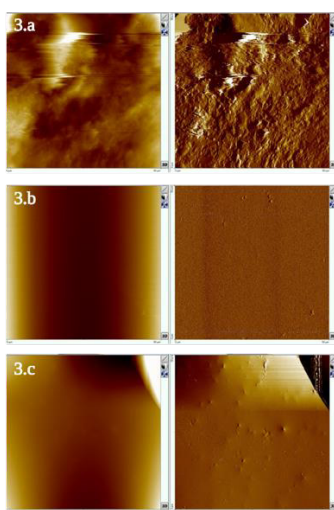


Figure 3. a) untreated Tempranillo must, b) UHPH treated Tempranillo must (2400 bar), and c) UHPH treated must (3000 bar).

There is no doubt that the nano structure has been also modified. The images done with the nano observer AFM made possible to measure the particles observed as small lumps in the treated musts, while untreated musts appear to have larger number of lumps and higher roughness. The darker and brighter areas observed in Figs. 3a and 4a is a direct sign of the size of the particles.

The average particle size for those pimple structures was measured with the nano observer AFM for both musts, treated and untreated. In the case of Tempranillo, the average value for fresh untreated must was μm , while the UHPH treated musts had μm and μm for 2400 bar and 3000 bar respectively. Regarding the Verdejo juice, the average values obtained for the particle size were μm for untreated fresh must and μm for the 3000 bar UHPH treated juice.

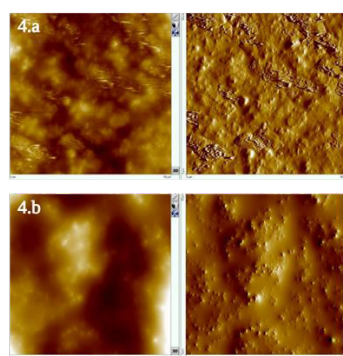


Figure 4. a) untreated Verdejo must, b) UHPH treated Verdejo must.

These observations are contrasted with other technique that is able to measure the size of particles such as the laser diffraction.

The laser diffraction has shown what the particle size of a treated must is. In particular, the parameters D50 and D90 which establish that 50% and 90% of the particles, have a size smaller than $0.276 \pm 0.015 \mu\text{m}$ and $2.727 \pm 0.373 \mu\text{m}$. Knowing this information, the possibility of finding yeast cells in musts treated with UHPH is drastically reduced as they usually have sizes similar to or larger than $8 \mu\text{m}$ at growing temperature of 20°C for single mother cells [11]. Figure 5 shows the distribution obtained for the particle size of a must treated with UHPH and analysed with a laser diffraction instrument.

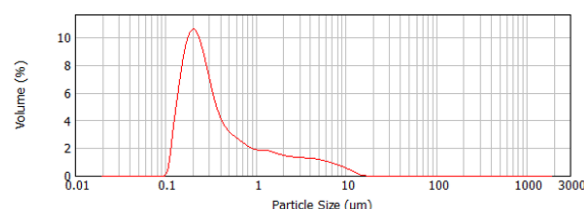


Figure 5. Laser diffraction of UHPH treated must showing the distribution of the particle size in microns.

Lastly, Table 1 summarizes the main oenological parameters which include reducing sugars (Glu + Fru) and dissolve solids (degrees Brix), pH, tartaric acid, and organic and inorganic sources of yeast asimilable

nitrogen (amino primary nitrogen and ammonium salts). The chemical composition determined with FTIR revealed no difference in these parameters between untreated and treated musts. Similar observations were obtained in previous experiments where Hondarribi zuri, *Vitis vinifera* L. white variety, was processed with UHPH and evaluated [12].

Table 1. Main oenological parameters determined with FTIR in untreated and treated musts.

ID		Verdejo		Tempranillo	
		Control	UHPH	Control	UHPH
Glu + Fru	g/L	137.8	140.8	237.9	236.9
Refract. (20°)	°Brix	15.2	15.4	24.0	23.9
pH		3.61	3.63	4.07	4.08
Tartaric acid	g/L	4.3	4.1	7.02	6.52
Amino Nitrogen	mg/L	81.5	93.2	149.8	138.4
Ammonium	mg/L	24.9	26.3	91.3	102.6

In this way, UHPH is a real alternative to the use of conventional antimicrobial additives to reduce microbial populations in musts, and to preserve chemical, sensorial and nutritional components of musts.

4 Conclusions

Ultra-high pressure homogenization is an emerging non-thermal technology that effectively eliminates the microbiota in juices from grapes of *Vitis vinifera* varieties. The absence of viable yeast cells is evinced through imaging with optical microscopy, and so was the modification of the structure at microscopic scale. The size of the particles in the juices is possible to be measured with the use of atomic force microscopy and laser diffraction. The results confirm the feasibility of these techniques to characterize treated and untreated

grape juices, and are, at the same time, a way to confirm the extent that UHPH has produced on this matrix. Lastly, the chemical composition of fresh treated and untreated musts confirm that these parameters are not modified after the treatment with UHPH.

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