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ULTRASONIC VELOCITY OF WATER – ETHANOL - MALIC ACID - LACTIC ACID MIXTURES DURING THE MALOLACTIC FERMENTATION PROCESS

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Abstract

During malolactic fermentation in wines, malic acid is transformed into lactic acid by the action of lactic acid bacteria. This process can be monitored on-line by measuring the velocity of a low intensity ultrasonic wave propagating through the medium. In this work, an experimental study of ultrasonic propagation velocity in laboratory mixtures of water - ethanol - malic acid and lactic acid is presented. A good correlation was found between the ultrasonic velocity and malic
and lactic acid concentrations. These results could be used to predict the end-point of the malolactic fermentation process and show the great potential of this ultrasonic technique to determine malic and lactic acid concentrations during the malolactic fermentation process.

*Keywords: Malolactic fermentation; Ultrasound; Process monitoring; Lactic acid; Malic acid.*

1- Introduction

Malolactic fermentation (MLF) is a process that consists in the transformation of malic acid into both lactic acid and carbon dioxide. This process, caused by lactic acid bacteria (LAB), takes place during the production of the majority of red wines as well as when producing certain types of white wines. The contribution of MLF is vital to the development of the sensory characteristics of wine: it reduces acidity, it adds microbiological stability and it improves the organoleptic profile by producing a wide range of colours, flavours and aromas (Wibowo et al., 1985; Maicas et al., 1999; Liu, 2002; Lerm et al., 2010).

During the winemaking process, MLF may be produced spontaneously due to the presence of lactic acid bacteria on the surface of the grapes. This may result in a lack of control over the malolactic stage and interferes with other stages, with uncertain results in the wine characteristics.

In order to obtain a correct, controlled malolactic fermentation process, a method known as “induced MLF” was recently introduced, consisting in the systematic inoculation of natural strains of LAB and the efficient monitoring of the MLF that ensues. However, the success of such an induced MLF is not guaranteed in all cases. The task of monitoring the progress of MLF is mostly carried out by measuring the concentration of malic and lactic acids in wine samples. Several measurement methods such as Paper Chromatography (PC), Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), enzymatic analysis, Fourier-transform Infrared Spectroscopy (FT-IR) and reflectance are described in the
literature (Lerm et al., 2010). Most of these methods, however, share the fact that they are both destructive and, rather complex. Moreover, when these methods are used, obtaining accurate results tends to be a rather time consuming process. On top of all of that, the methods themselves are, generally speaking, not affordable to small wineries.

Ultrasound is an emerging and promising technology for both wine processing and property sensing, at present mostly limited to research activities within a laboratory environment (Cortada et al., 2011; Jiranek et al., 2008; Lamberti et al., 2009; Salazar et al., 2009). As a novelty, an ultrasonic technique is proposed here to be used as an in-situ method for the on-line monitoring of the MLF progress. Unlike the conventional methods above, ultrasonic techniques are non-invasive, non-destructive, accurate, rapid, non-expensive, on-line and suitable for process automation (McClements, 1997). Having said that, these techniques are known to be highly sensitive to physical parameters such as temperature, an aspect that can sometimes act as a disadvantage.

The purpose of this paper is to study the ultrasonic propagation velocity of a 1 MHz sine-wave tone burst in laboratory mixtures of water - ethanol - malic acid and lactic acid, and the interactions between malic and lactic acids concentrations. A change in concentration of any of these components is seen to result in a change in the ultrasonic propagation velocity.

Experimental results show a good correlation between ultrasonic propagation velocity and the concentration of malic and lactic acids. Considering the overall costs of wine production management and control in terms of manpower, sampling and chemical analyses, the proposed system could represent an attractive solution for the on-line monitoring of malolactic fermentation processes. In addition, the ultrasonic velocity could also be used to predict the end of the malolactic fermentation process. The experimental method used, the difficulties encountered along the way as well as the results obtained are also discussed in this paper.

2- Materials and methods.
2.1 Malolactic fermentation (MLF).

2.1.1. Stoichiometry.

As shown in Eq. 1, during MLF malic acid \((\text{C}_4\text{H}_6\text{O}_5)\) is transformed to lactic acid \((\text{C}_3\text{H}_6\text{O}_3)\) and carbon dioxide \((\text{CO}_2)\). This process is catalysed by a highly specialised enzyme (the “malolactic enzyme”) and carried out by the LAB, mainly those of *Oenococcus, Lactobacillus, Pediococcus* and *Leuconostoc* strains (Wibowo et al., 1985).

\[
\text{C}_4\text{H}_6\text{O}_5 \rightarrow \text{C}_3\text{H}_6\text{O}_3 + \text{CO}_2 \quad \text{Eq. 1}
\]

Stoichiometrically, 1 mole of malic acid produces 1 mole of lactic acid and 1 mole of carbon dioxide. But if mass concentrations are considered, the relationship between malic acid and lactic acid in an MLF process is described by Eq. 2

\[
x_{\text{lactic acid}} = x_{\text{lactic acid}}^0 - \frac{M_{\text{lactic acid}}}{M_{\text{malic acid}}} (x_{\text{malic acid}} - x_{\text{malic acid}}^0) \quad \text{Eq. 2}
\]

In Eq. 2, \(x_i\) refers to mass concentration of component \(i\), superscript \(^0\) refers to the beginning of the fermentation and \(M_i\) represents the molar mass of component \(i\).

The molar masses of lactic acid and malic acid are 90.08 g/mole and 134.09 g/mole, respectively. According to this, the ratio of these molar masses is approximately 0.67. So, considering Eq. 2, during the MLF process, a 3 g/l reduction of malic acid equals to an increase of about 2 g/l of lactic acid.

2.1.2. MLF process.

Three steps are defined in MLF, correlative in time: (i) bacterial growth phase, (ii) stationary phase I and (iii) stationary phase II (Krieger, 2006).

(i) Bacterial growth phase:
This phase starts when lactic acid bacteria (LAB) are inoculated. LAB growth takes place during this phase. This results in a consumption of sugars that were no fermented during the alcoholic fermentation phase. A slight amount of acetic acid is also produced. No malic acid is metabolized, so malic acid and lactic acid concentrations are stable.

(ii) Stationary phase I:

This phase starts when the bacterial growth phase is finalized. During this phase, the amount of LAB is stable and malic acid is transformed to lactic acid. No sugar consumption is produced (LAB prefer malic acid).

(iii) Stationary phase II:

This is the last phase. During this third phase, no more malic acid is transformed to lactic acid, but citric acid is degraded and acetic acid is produced. Also, the amount of LAB is reduced. This phase should be avoided in wineries, because wine characteristics are degraded.

So, it is important to determine the end point of phase (ii), in order to prevent phase (iii) from happening.

2.1.3. Control of MLF.

Decarboxylation of the malic acid in wine is the most obvious action of the MLF. The easiest way to monitor the progress of the MLF is to chemically analyze the disappearance of malic acid and the formation of lactic acid. The most commonly used quantitative analytical method for monitoring MLF is the enzymatic determination of L-malic acid. This method uses an enzyme that specifically reacts with L-malic acid and a UV-visible spectrophotometer to monitor the progress of the analytical reaction. Kits from manufacturers that contain all the reagents, enzymes and procedures required for L-malic acid determinations are readily available. For this study a multiparametric analyzer Lisa 200 (Hycel diagnostics, TDI Tecnología Difusión Ibérica, S.L., Spain) was used. In addition, two separate kits were used,
one for each reagent: an L-Malic Acid Enzymatic Kit (Boehringer Mannheim-Roche, Spain) and an L-Lactic Acid Enzymatic Kit (Boehringer Mannheim-Roche, Spain). The detection of L-malic acid requires two enzyme reactions. In the first reaction, malic acid (L-malate) is oxidized to oxaloacetate by nicotinamide-adenine dinucleotide (NAD) in the presence of L-malate dehydrogenase (L-MDH):

\[
L\text{-Malate} + NAD^+ \overset{L\text{-MDH}}{\leftrightarrow} \text{oxaloacetate} + NADH + H^+ \quad \text{Eq. 3}
\]

However, since the equilibrium of reaction (Eq. 3) lies firmly in the favour of L-malate and NAD\(^+\), a further reaction is required to trap the NADH product, and this is achieved by the conversion of oxaloacetate to L-aspartate and 2-oxoglutarate, in the presence of a large excess of L-glutamate, by glutamate-oxaloacetate transaminase (GOT):

\[
\text{Oxaloacetate} + \text{L-glutamate} \overset{\text{GOT}}{\leftrightarrow} \text{L-aspartate} + 2\text{-oxoglutarate} \quad \text{Eq. 4}
\]

The amount of NADH formed is stoichiometric to the amount of L-malate. The increase in NADH is measured through the measurement of its light absorbance at 334, 340 or 365 nm.

### 2.2. Ultrasonic velocity in liquid media.

When the distance travelled by an ultrasonic wave through a liquid medium is a known constant, the wave’s velocity can be calculated using Eq. 5

\[
v = \frac{d_{\text{travelled}}}{\text{TOF}} \quad \text{Eq. 5}
\]

where TOF corresponds to the time of flight, which is the time taken by a wave to travel a given distance (\(d_{\text{travelled}}\)). A series of practical methods to measure TOF were described and analyzed in a previous paper (Novoa-Díaz et al., 2012), as was the method for determining ultrasonic velocity.
Generally, TOF varies in accordance with the physical and chemical changes in the medium. Given this, it is reasonable to assume that variations of lactic and malic acid concentrations in the liquid mixture will cause changes to the TOF, and consequently, to the ultrasonic wave velocity. Generally speaking, the propagation parameters for ultrasonic waves in the medium are a composite of the separate contributions made by each individual element present in the medium (Resa et al., 2007).

2.3 Reagents.

Reagents used to prepare the mixtures were distilled water, Ethanol 96% v/v PA-ACS (Panreac Química S.L.U., Barcelona, Catalonia, Spain), DL-Malic acid (purity >99%, Sigma-Aldrich Co, St. Louis, MO, USA) and L(+)-Lactic Acid (purity >95%, Panreac Química S.L.U., Barcelona, Catalonia, Spain).

2.4. Mixtures.

An ethanol 11.5% solution has been prepared from Ethanol 96% using 1,000ml and 100ml volumetric flasks and 10ml pipettes (Duran, Germany). A malic acid concentrated solution (0.25 g/l) was prepared from reagents by weighing the solute using a Cobos CB-Complet digital scale with a precision of 0.001 g. Discrete amounts of malic acid (0.25 g/l) and lactic acid (purity >95%) were added to samples of distilled water or ethanol 11.5% solution by using a 1,000 µl precision microliter pipette (P200 Gilson’s PIPETMAN P, Gilson, Villiers-le-Bel, France), until their complete dissolution.

2.5 Experimental cell.

2.5.1. Aqueous solutions.
Samples of malic and lactic acids solved in distilled water were placed in a 600 ml glass beaker, which was immersed in a thermostatic bath at 22.5° ± 0.1°C (Omega, Stamford, UK). An ultrasonic transducer (B1F, General Electric, USA) was connected to a buffer rod and placed in contact with the aqueous sample, as shown in Fig. 1. The temperature of the solutions is measured using a Fluke 1551A Pt100 Thermometer, with a 0.05°C precision.

2.5.2. Hydroalcoholic solutions.

In order to measure ultrasonic velocity in hydroalcoholic solutions, two identical ultrasonic transducers (B1F, General Electric, USA) were used. One of them was placed into a 600 ml glass beaker, filled with samples of malic and lactic acids solved in ethanol 11.5%. The other one was placed into an identical 600 ml glass beaker, filled only with an ethanol 11.5% solution. No malic acid or lactic acid samples were placed in this second glass beaker. In this case, two ultrasonic transducers were used in order to evaluate and compensate for the ethanol evaporation effect, which causes severe changes to ultrasonic velocity. As in section 2.5.1, both glass beakers were immersed in a thermostatic bath at 22.5° ± 0.1°C (Omega, Stamford, UK), see Fig. 1.

2.5 Ultrasonic velocity measurement.

The method for the determination of the ultrasonic velocity was based on a pulse-echo technique using a tone-burst pulse. An emitter-receiver ultrasound transducer, attached to a cylindrical buffer rod, was excited at its 1 MHz resonant frequency with a sine-wave tone burst of 10 cycles and 20 Vpp of amplitude, using an Agilent 33522 function/Arbitrary Waveform Generator. The generated acoustic wave propagates along the buffer rod until it reaches the buffer rod-liquid interface. Then, part of the incident wave is reflected back to the ultrasonic transducer (ECHO1) and the other part is transmitted through the liquid sample until it reaches
the surface of an acoustic reflector. At the reflector, the transmitted wave is reflected back towards the liquid-buffer rod interface, where once again part of this signal is transmitted through the buffer rod and detected by the ultrasound transducer (ECHO2). The received waves were acquired using a Tektronix 200 MHz / 1Gs/s DPO 2024 Digital Phosphor Oscilloscope (Fig. 2).

Finally, the acquired signals were analyzed using a fast Fourier transform (FFT) algorithm to obtain the time of flight in the liquid (TOF$_{\text{liquid}}$), as described in previous work (Novoa-Díaz et al., 2012). Then, the ultrasonic propagation velocity of a wave in the liquid was calculated by dividing the travelled distance through the liquid by the time of flight, as stated in Eq. 5. The ultrasonic propagation velocity was calculated with an uncertainty of less than ±0.1 m/s (Novoa-Díaz, 2014).

3- Results and discussion.

3.1. Influence of solute concentrations on ultrasonic velocity.

In order to determine the influence of the different factors on the ultrasonic propagation velocity, a set of experiments were designed, or more specifically a $2^3$ factorial experiment. Factors considered were the following: malic acid concentration, lactic acid concentration and ethanol concentration. The temperature factor was kept constant. So, a set of 8 experiments were designed, as a result of combining the three considered factors with two concentration levels (high and low). For each experiment, three measurements were performed.

Experimental results are shown in Table 1. The ultrasonic propagation velocity is the response and, factors a, b and c correspond to the malic acid, lactic acid and ethanol concentrations respectively. The two levels considered were: low level (with zero concentration of the respective factors) and high level (6 g/l of malic and lactic acid concentration, 11.5% v/v of ethanol concentration). These values of velocity are higher than those generally found in pure
water (Grosso and Mader, 1972) and within the range of the velocity values reported for water-ethanol mixtures (Vatandas et al., 2007).

Carrying out a variance analysis (ANOVA) on the experimental results shown in Table 1, it is possible to evaluate the influence of the different factor effects on the ultrasonic propagation velocity.

Results of ANOVA for the aforementioned factorial experiment are shown in Table 2. A, B and C correspond to the three considered factors (malic acid concentration, lactic acid concentration and ethanol concentration, respectively). AB, AC, BC and ABC correspond to the interaction effects between the factors.

From results shown in Table 2 we can conclude:

1- From the three considered factors, the most significant is ethanol concentration. It is therefore crucial to keep this factor under control when ultrasonic propagation velocity in hydroalcoholic solutions is measured. This fact will determine the measurement technique to be used, as explained in section 3.3 of this paper.

2- Factors A and B (malic and lactic acid concentrations, respectively) are less significant than factor C (ethanol concentration). The ultrasonic propagation velocity varies little with the concentration of these acids. Therefore, it is important to avail of sufficient measurement accuracy in order to be able to observe any changes.

3- A very significant interaction between factors A (malic acid concentration) and C (ethanol concentration) is observed. The ultrasonic propagation velocity is directly proportional to malic acid concentration in aqueous solutions, in contrast to what happens in hydroalcoholic samples. In the latter case, the ultrasonic propagation velocity is inversely proportional to the concentration of malic acid. However, the
interaction between lactic acid and ethanol concentration (interaction BC) is less
significant than the AC interaction, so the ultrasonic propagation velocity increases
with the lactic acid concentration (both in aqueous and hydroalcoholic solutions).

4- No significant interaction between factors A and B is observed. Thus, a linear response
of the ultrasonic velocity propagation with respect to lactic and malic acid
concentrations is expected.

3.2. Ultrasonic velocity in hydroalcoholic solutions.

3.2.1. Preliminary.

As discussed in section 3.1, the ethanol factor predominates over the malic and lactic acid
factors. As a result, very small variations of ethanol concentration can easily mask ultrasonic
velocity variations due to changes in malic or lactic acids concentrations. For this reason, it is
very important to keep ethanol concentration constant during the whole experimental process.
Should this not be possible, ultrasonic velocity variations due to changes in ethanol
collection should be kept under control.

During the experimental process, it was found that it was possible for some of the ethanol to
evaporate, due to the fact that the samples were placed in a glass beaker that was not sealed.
This has an effect on the experimental results. Since keeping the experimental cell completely
sealed was not a viable option, it was decided to use an experimental cell based on two identical
sensors: the first sensor (sample) measured the ultrasonic propagation velocity in a
hydroalcoholic solution of malic and lactic acids. The second sensor (blank) measured the
ultrasonic propagation velocity in the same hydroalcoholic solution but with no added malic or
lactic acids. Both sensors were previously calibrated in order to guarantee that the same results
were obtained in identical samples. Fig. 3 shows the experimental cell used.
3.2.2. Ternary mixtures water - ethanol- malic acid.

The ultrasonic propagation velocity in hydroalcoholic solutions of malic acid is shown in Fig. 4, where aliquots of malic acid were gradually added to the water in the flask where the ultrasonic sensor was placed. Samples were thermostated to 22.20 ± 0.05 ºC. Malic acid concentration varies between 0 g/l to 10 g/l. A linear behavior is observed, were the slope is -0.200 m/s per g/l of malic acid, and the correlation coefficient $R^2$ is 0.9988.

As indicated in section 3.1, ultrasonic velocity decreases as malic acid concentration in hydroalcoholic solutions increases, in contrast to aqueous solutions (were ultrasonic velocity is directly proportional to malic acid concentration). One possible explanation would be that the malic acid reacts with the ethanol. Consequently, an esterification between malic acid and ethanol takes place, resulting in ethyl malate and water being formed. This implies that the ethanol concentration is reduced as water concentration increases. Due to the fact that the ultrasonic propagation velocity is slower in water than in ethanol 11.5% v/v, the esterification causes a decrease in ultrasonic velocity, which is proportional to the increase in malic acid concentration.

3.2.3. Ternary mixtures water - ethanol- lactic acid.

Similarly, the ultrasonic velocity of hydroalcoholic samples of lactic acid was measured, thermostated to 22.20 ± 0.05 ºC (Fig. 5).

From results shown in Fig. 5, a linear behavior between ultrasonic propagation velocity and lactic acid concentration is observed. The slope is 0.243 m/s per g/l of lactic acid (slightly lower than the value obtained in aqueous solutions).

It should be noted that in this case the ultrasonic velocity increases with lactic acid concentration, as opposed to malic acid samples in hydroalcoholic solutions. This may be due to the fact that, in case of lactic acid hydroalcoholic solutions, the esterification is more difficult,
because the lactic acid is polymerized. As a result, the ultrasonic velocity behavior in hydroalcoholic solutions is similar to the behavior in aqueous solutions.

3.2.4. Quaternary mixtures water - ethanol- malic acid - lactic acid.

Finally, ultrasonic velocity in quaternary mixtures water – ethanol - lactic acid – malic acid was measured. Thermostated samples of malic acid in different concentration (0, 2, 4, 6, 8 and 10 g/l), solved in ethanol 11.5% v/v, were prepared. Aliquots of lactic acid were added to the samples of malic acid, and the ultrasonic propagation velocity was measured. Results are represented graphically in Fig. 6.

Empirical equations from the data obtained in Fig. 6 have been derived, using a linear model. The derived equations are shown in Table 3.

In Table 3, a good fit is observed between the empirical equations and experimental data, with a correlation coefficient $R^2$ higher than 0.99 in all of them. The linear behavior is consistent with the observed non-interaction between both acids (malic and lactic), as indicated in section 3.1.

From data represented in Fig. 6 it is possible to obtain a 3D graph that correlates the ultrasonic velocity with malic and lactic acid concentrations (Fig. 7).

From data represented in Fig. 7, a linear empirical equation has been derived (Eq. 6).

$$v = 1556.45 - 0.2196 \cdot x_{\text{malic acid}} + 0.2359 \cdot x_{\text{lactic acid}} \quad \text{Eq. 6}$$

In Eq. 6, $v$ refers to ultrasonic velocity (in m/s), $x_{\text{malic acid}}$ corresponds to malic acid concentration (in g/l) and $x_{\text{lactic acid}}$ corresponds to lactic acid concentration (in g/l). The correlation coefficient $R^2$ is 0.996.

As shown in Eq.1, during MLF malic acid concentration decreases and lactic acid increases. So, according to Eq. 6, ultrasonic velocity should increase as MLF takes place.
3.3. Ultrasonic velocity in wine samples.

To conclude, ultrasonic velocity in real wine samples is measured. LAB are inoculated in a tempranillo wine sample in order to induce the MLF, and ultrasonic velocity is monitored (Fig. 8). An increase of ultrasonic velocity as MLF takes places is observed. This is consistent with ultrasonic velocity data obtained in the laboratory, as indicated in section 3.2.4 of this paper (Fig. 7 and Eq. 6)

Ultrasonic velocity is highly dependent on temperature. In a recent work (García-Álvarez et al., 2011), the strong dependence on temperature of ultrasonic velocity in red wine samples was shown. It is clear that thermostated measurements are rather difficult to perform in wineries. For this reason, temperature was monitored during all the experiments and the temperature dependency of ultrasonic velocity was compensated for accordingly.

Unfortunately, ultrasonic velocity depends not only on temperature but also on alcohol concentration. In fact, the dependence of ultrasonic velocity on temperature is in turn dependent on alcohol concentration. Therefore, the ultrasonic propagation velocity in hydroalcoholic samples at different temperatures and for different concentration values of ethanol solution was measured. From these results and, for different values of ethanol concentration, it was possible to derive the amount of change in ultrasonic propagation velocity for every degree of change in temperature, as Fig. 9 depicts.

In order to obtain the concentrations of malic and lactic acids the following procedure is followed. As a first approximation, the 4 factors considered in this work are malic and lactic acids, ethanol and temperature. From the stoichiometric equation, there is a relationship between malic and lactic acids. Thus, a 3g/l reduction in malic acid equals to an increase of 2 g/l in lactic acid. In addition, there is no interaction between malic and lactic acids (section 3.1). Thus, the contribution of each acid to ultrasonic velocity can be derived separately. However, the effect of ethanol and temperature must be taken into account first. To this effect, the
temperature compensation coefficient for the ultrasonic velocity (m/s per °C) can be determined from Fig. 9, for a given wine alcohol level.

Fig. 8 also shows temperature variation during MLF and temperature compensated ultrasonic velocity variation. The results obtained show that the ultrasonic velocity variation initially increases, followed by a decrease and ending up increasing again until a new stable value is reached, which is higher than the initial value. It is also observed that the ultrasonic velocity variation calculated after applying a temperature compensation coefficient is significantly lower than the measured value.

The aim of the method under study is to determine the concentrations of malic and lactic acids using ultrasonic velocity measurements (and to estimate the end point of MLF). So, from the temperature compensated ultrasonic velocity plot obtained in Fig. 8, and by applying Eq. 2 and Eq. 6, it is possible to estimate the variation of malic and lactic acid concentrations (Fig. 10). These results will be then compared with the malic and lactic acid concentrations obtained using a multiparametric analyser such as a Lisa 200 (Hycel diagnostics, TDI Tecnología Difusión Ibérica, S.L., Spain) by enzymatic methods.

In Fig. 10, the estimated concentrations are plotted starting at a time of 100h (approximately), but not before. The reason for this decision is that the ultrasonic velocity variation in wine samples before this period of time is not due to changes in lactic and malic acid concentrations, but to different factors. Namely, according to section 2.1.2, the bacterial growth phase (i) takes place at the beginning of MLF. In this phase, malic acid is not transformed to lactic acid, but other processes take place that result in changes in ultrasonic velocity. Typically, the bacterial growth phase (i) occurs from the beginning of MLF to a time of 72-96 h. After the bacterial growth phase (i), the stationary phase I (ii) takes place, when malic acid is turned into lactic acid. This happens during the period ranging from 100h to 196-216h in our experiment. The time of 100h is considered as a threshold value, because it matches with the minimum
increment value of ultrasonic propagation velocity after temperature compensation, as shown in Fig. 8.

According to the previous paragraph, the results obtained from ultrasonic velocity measurements during the bacterial growth phase (i) should not be considered. After this phase (i), the stationary phase I (ii) takes place, when malic acid is transformed into lactic acid. In this phase the ultrasonic velocity variation is due mainly to changes in lactic and malic acid concentrations, and consequently, during this period of time the measured and estimated values of concentration agree (as shown in Fig 10).

As a conclusion, during stationary phase I a good correlation between measured and estimated values is observed (Fig. 10), a fact that points to the suitability of this technique for monitoring the fermentation during this period of time.

4- Conclusions.

This work shows the suitability of measuring ultrasonic propagation velocity as an input to monitoring on-line malolactic fermentation processes, with a good correlation between ultrasonic velocity and malic and lactic acid concentrations solved in ethanol 11.5% v/v. During malolactic fermentation, malic acid is transformed to lactic acid, and that causes an increase of the ultrasonic velocity of propagation. Consequently, ultrasonic velocity can be used to predict the end-point of the malolactic fermentation process.

It is important to point out that ultrasonic velocity is highly correlated to both ethanol concentration and temperature. Therefore, it is critical to both control those parameters and compensate for their variations if a good monitoring of the malolactic fermentation process is desired. Also, in real wine samples, the malic acid is transformed to lactic acid only after an initial phase (bacterial growth phase (i)), a phase which should be excluded from the measurements.
Malolactic fermentation is a very complex process. This paper mostly reflects the state of a work in progress and reports on the current status of the research being carried out by the authors. In order to be able to develop a future commercial prototype, further studies need to be carried out, especially on real wine samples, taking into account all of the factors mentioned above.

5- Acknowledgement.

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The authors would like to thank Daniel Carles for his help and valuable comments on this paper.

6- References.


Fig. 1. Experimental set-up.
Fig. 2. Ultrasonic velocity measurement. \( \text{TOF}_{\text{BR}} \) : time-of-flight in the buffer rod. \( \text{TOF}_{\text{liquid}} \) : time-of-flight in the liquid solution.
Fig. 3. Experimental cell used for measure ultrasonic propagation velocity in hidroalcoholic solutions of malic and lactic acids.
Fig. 4. Ultrasonic velocity of propagation in ternary mixtures of water-ethanol-malic acid, thermostated to $22.20 \pm 0.05 \, ^\circ C$. 

\[ y = 0.72x + 1556.4 \]

\[ R^2 = 0.9988 \]
Fig. 5. Ultrasonic velocity in ternary mixtures of water-ethanol-lactic acid thermostated to 22.20 ± 0.05 °C.
Fig. 6. Ultrasonic velocity in quaternary mixtures of water-ethanol 11.5% v/v-lactic acid-malic acid, thermostated to 22.20 ± 0.05 °C.
Fig. 7. 3D graph representation of ultrasonic velocity in quaternary mixtures of water-ethanol 11.5% v/v-lactic acid-malic acid, thermostated to 22.20 ± 0.05 °C.
Fig. 8. Ultrasonic velocity variation and temperature variation, during MLF of a tempranillo wine sample. Δvel corresponds to velocity variation measured, Δvel-compT refers to velocity variation after temperature compensation and Δt refers to temperature variation.
Fig. 9. Ultrasonic velocity increment (in m/s) due to an increment of a 1ºC as a function of the concentration of ethanol in an aqueous solution.
Fig. 10. Variation of malic and lactic acid concentration versus time. $\Delta$malic-est and $\Delta$lactic-est refer to estimate values obtained from ultrasonic velocity. $\Delta$malic-meas and $\Delta$lactic-meas correspond to measured values using the analytical reference method (Enzymatic method).
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<td>1,482.85</td>
</tr>
<tr>
<td>c</td>
<td>1,556.43</td>
<td>1,556.44</td>
<td>1,556.37</td>
<td>1,556.41</td>
</tr>
<tr>
<td>ac</td>
<td>1,555.19</td>
<td>1,555.24</td>
<td>1,555.13</td>
<td>1,555.19</td>
</tr>
<tr>
<td>bc</td>
<td>1,557.87</td>
<td>1,557.87</td>
<td>1,557.73</td>
<td>1,557.82</td>
</tr>
<tr>
<td>abc</td>
<td>1,556.53</td>
<td>1,556.58</td>
<td>1,556.49</td>
<td>1,556.53</td>
</tr>
</tbody>
</table>

Table 1. Ultrasonic velocity propagation results for a 2^3 factorial experiment. (I) – blank, a – malic acid concentration, b – lactic acid concentration, c – ethanol concentration.
<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares (SS)</th>
<th>Degrees of freedom</th>
<th>Mean Square (MS)</th>
<th>(F_0)</th>
<th>(F_{0.01,1,16})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.4928</td>
<td>1</td>
<td>0.4928</td>
<td>311.57</td>
<td>8.53</td>
</tr>
<tr>
<td>B</td>
<td>15.5118</td>
<td>1</td>
<td>15.5118</td>
<td>9,806.78</td>
<td>8.53</td>
</tr>
<tr>
<td>C</td>
<td>34,218.8745</td>
<td>1</td>
<td>34,218.8745</td>
<td>21,633,702.93</td>
<td>8.53</td>
</tr>
<tr>
<td>AB</td>
<td>0.0058</td>
<td>1</td>
<td>0.0058</td>
<td>3.65</td>
<td>8.53</td>
</tr>
<tr>
<td>AC</td>
<td>14.2090</td>
<td>1</td>
<td>14.2090</td>
<td>8,983.18</td>
<td>8.53</td>
</tr>
<tr>
<td>BC</td>
<td>0.3185</td>
<td>1</td>
<td>0.3185</td>
<td>201.34</td>
<td>8.53</td>
</tr>
<tr>
<td>ABC</td>
<td>0.0243</td>
<td>1</td>
<td>0.0243</td>
<td>15.36</td>
<td>8.53</td>
</tr>
<tr>
<td>Error</td>
<td>0.0253</td>
<td>16</td>
<td>0.0016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>34,249.4620</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. ANOVA for results obtained in table 1. A – malic acid concentration, B – lactic acid concentration, C – ethanol concentration.
<table>
<thead>
<tr>
<th>Malic Acid Concentration</th>
<th>Empirical function</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 g/l</td>
<td>$y = 0.2432x + 1556.4$</td>
<td>0.9999</td>
</tr>
<tr>
<td>4 g/l</td>
<td>$y = 0.2425x + 1555.9$</td>
<td>0.9960</td>
</tr>
<tr>
<td>6 g/l</td>
<td>$y = 0.2381x + 1555.6$</td>
<td>0.9950</td>
</tr>
<tr>
<td>8 g/l</td>
<td>$y = 0.2427x + 1555.1$</td>
<td>0.9960</td>
</tr>
<tr>
<td>10 g/l</td>
<td>$y = 0.2275x + 1554.8$</td>
<td>0.9980</td>
</tr>
</tbody>
</table>

Table 3. Adjusted empirical functions and correlation coefficient $R^2$, for ultrasonic velocity (y-axis) related to lactic-acid concentration (x-axis), for 11.5 % v/v hydroalcoholic samples of malic acid. Units: x (g/l), y (m/s).
Highlights.

The measurement of the velocity of an ultrasonic pulse may be used to monitor the malolactic fermentation process.

Significant correlations were observed between ultrasonic velocity and malic and lactic acid concentrations in laboratory aqueous and hydroalcoholic samples.

The ultrasonic velocity could be used to predict the end of the malolactic fermentation process.