INACTIVATION OF FOODBORNE PATHOGENS AND ENZYMES BY ULTRASOUND UNDER PRESSURE AT NON-LETHAL AND LETHAL TEMPERATURES IN APPLE AND ORANGE JUICES

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submitted by **BURÇİN HÜLYA GÜZEL** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Food Engineering Department, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Alev BAYINDIRLI _____
Head of Department, **Food Engineering**

Prof. Dr. Hami ALPAS _____
Supervisor, **Food Engineering Dept., METU**

Prof. Dr. Alev BAYINDIRLI _____
Co-Supervisor, **Food Engineering Dept., METU**

Examining Committee Members

Prof. Dr. Faruk BOZOĞLU _____
Food Engineering Dept., METU

Prof. Dr. Hami ALPAS _____
Food Engineering Dept., METU

Prof. Dr. Vural GÖKMEN _____
Food Engineering Dept., Hacettepe University

Assoc. Prof. Dr. Behiç MERT _____
Food Engineering Dept., METU

Assist. Prof. Dr. Deniz ÇEKMECELİOĞLU _____
Food Engineering Dept., METU

Date: September 4th, 2013

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct. I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Burçin Hülya, Güzel

Signature:

ABSTRACT

INACTIVATION OF FOODBORNE PATHOGENS AND ENZYMES BY ULTRASOUND UNDER PRESSURE AT NON-LETHAL AND LETHAL TEMPERATURES IN APPLE AND ORANGE JUICES

Güzel, Burçin Hülya
Ph.D., Department of Food Engineering
Supervisor : Prof. Dr. Hami Alpas
Co-Supervisor : Prof. Dr. Alev Bayındırlı

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The inactivation of *Listeria monocytogenes* and *Escherichia coli* suspended in UHT treated apple and orange juices by ultrasound under pressure at nonlethal (Manosonication-MS) and lethal temperatures (Manothermosonication-MTS) was evaluated. Significant differences were found among the MS resistance (35 °C, 110 μm, 200 kPa) of five strains of *L. monocytogenes* and three of *E. coli* in pH 3.5 buffer, being *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 the most resistant strains. Regarding the interspecific differences, *L. monocytogenes* showed higher MS resistance than *E. coli*. Although pH and treatment medium composition did not significantly change the bacterial MS resistance, the effectiveness of ultrasound increased by both raising the amplitude of ultrasonic waves and the pressure. The energy transmitted to the fruit juices by ultrasonic waves at different combinations of amplitudes (46.5, 90, 110, and 130.5 μm) and pressures (0, 100, and 200 kPa) was also studied, obtaining an exponential relationship between D_{MS} values and power input: an increase of 116 W increased the inactivation rate approximately 10-fold in both juices. The MS resistance of both species decreased when heat was applied jointly with ultrasound (MTS), which was more effective on inactivating *L. monocytogenes* and *E. coli* than the sum of MS and heat acting simultaneously but independently. Therefore, MTS showed a synergistic lethal effect in acidic juices, whose magnitude was dependent on the treatment conditions.

The inactivation of polyphenoloxidase (PPO) in freshly squeezed apple juice and pectinmethlyesterase (PME) in freshly squeezed orange juice by ultrasound under pressure at nonlethal (MS) and lethal temperatures (MTS) was evaluated**.** The temperature profiles of enzyme inactivation and the energy transmission in the fruit juices by ultrasonic waves at different combinations of amplitudes (90, 110, 130.5 μm) and pressures (0, 100, 200 kPa) were also studied. Amplitude and pressure had significant effects ($p \leq 0.05$) on the final temperatures of acidic fruit juices. An exponential relationship between D_{MS} values and power input was obtained and an increase of 113 W in the energy transferred into the freshly squeezed apple and orange juices by US will

make the inactivation rate of PPO and PME increase by 10 times in both juices. Both increasing the amplitude and the pressure caused an increase in the lethality of MS treatment in both freshly squeezed fruit juices. The comparison of the inactivation by thermal treatment (TT) with the inactivation by MS treatment showed that the combined process (MTS) was more efficient on reduction of enzyme activity than TT acting alone. MS was less effective on the inactivation of PPO enzyme than heat and MTS. Thermal inactivation was more effective on the inactivation of PPO in apple juice during MTS; contrary to MS and MTS process, which were more effective on the inactivation of PME in orange juice than on the inactivation of heat, because of the high thermo-stability of PME. Overall, the ultrasonic resistance of PME in orange juice was much higher than the PPO in apple juice. According to treatment conditions, MTS treatment showed a synergistic lethal effect in the acidic fruit juices. The magnitude of the synergistic effect had almost the same values for both enzymes.

Keywords: ultrasound, manosonication, manothermosonication, *Listeria monocytogenes*, *Escherichia coli* O157:H7, polyphenoloxidase, pectinmethlyesterase, apple juice, orange juice.

ÖZ

NON-LETAL VE LETAL SICAKLIKLARDA BASINÇ ALTINDAKİ ULTRASESİN ELMA VE PORTAKAL SULARINDA GIDA KAYNAKLI PATOJENLERİN VE ENZİMLERİN İNAKTİVASYONUNA ETKİSİ

Güzel, Burçin Hülya
Doktora, Gıda Mühendisliği Bölümü
Tez Yöneticisi : Prof. Dr. Hami Alpas
Ortak Tez Yöneticisi : Prof. Dr. Alev Bayındırlı

Eylül 2013, 240 Sayfa

Öldürücü ve öldürücü olmayan sıcaklıklarda basınç altındaki ultrasesin UHT uygulanmış elma ve portakal sularında *Listeria monocytogenes* ve *Escherichia coli* inaktivasyonu üzerine etkileri değerlendirilmiştir. McIlvaine tampon çözeltisi (pH 3.5) içinde beş *L. monocytogenes* suşunun ve üç *E. coli* suşunun MS (35 °C, 110 µm, 200 kPa) dirençleri arasında belirgin farklılıklar bulunmuş ve en fazla MS direncine sahip olarak *L. monocytogenes* STCC 5672 ve *E. coli* O157:H7 suşları belirlenmiştir. Türler arası farklılıklarla ilişkili olarak, *L. monocytogenes* suşunun *E. coli* suşundan daha yüksek MS direnci gösterdiği gözlemlenmiştir. Bakteriyel MS direnci üzerine pH ve uygulama ortamı kompozisyonunun önemli bir etkisi olmamasına rağmen ultrases dalgalarının genliği ve basıncın her ikisininde artmasının ultrases etkinliğinin artmasına neden olduğu belirlenmiştir. Farklı genlik (46.5, 90, 110, 130.5 µm) ve basınç (0, 100, 200 kPa) kombinasyonuna sahip ultrases dalgalarının oluşturduğunu enerjinin asidik meyve sularından geçişi de bu çalışma içinde değerlendirilmiş ve D_{MS} değerleri ile güç girişi arasında üstel bir ilişki bulunmuştur. Güç girdisindeki 116 W'lık bir artış, iki meyve suyundaki mikroorganizma inaktivasyon oranını 10 kat artırmıştır. Ultrases ile birlikte ısı uygulanması (MTS) iki suşunda MS direncini azaltmıştır ve MTS'in *L. monocytogenes* ve *E. coli* inaktivasyonu üzerine etkisi MS ve ısı uygulamalarının aynı anda fakat bağımsız olarak uygulanmasıyla elde edilen inaktivasyon etkilerinin toplamından daha fazla olduğu gözlenmiştir. Bu yüzden, asidik meyve sularında MTS'in sinerjistik inaktive etkisi gösterdiği ve bu etkinin büyüklüğünün uygulama şartlarına bağlı olarak değiştiği belirlenmiştir.

Öldürücü ve öldürücü olamayan sıcaklıklarda basınç altındaki ultrasesin taze sıkılmış elma ve portakal sularında doğal olarak bulunan polifenoloksidaz (PPO) ve pektinmetilesteraz (PME) enzimlerinin inaktivasyonu üzerine etkileri değerlendirilmiştir. Farklı genlik (90, 110, 130.5 µm) ve basınç (0, 100, 200 kPa) kombinasyonuna sahip ultrases dalgalarının oluşturduğunu enerjinin asidik meyve sularından geçişi ve bu kombinasyonun enzim inaktivasyonu sırasında oluşturduğu sıcaklık profili de bu çalışma

içinde değerlendirilmiştir. Genlik ve basıncın asidik meyve sularının son sıcaklığı üzerine önemli etkileri gözlemlenmiştir. D_{MS} değerleri ile güç girişi arasında üstel bir ilişki bulunmuş ve güç girdisindeki 113 W'lık bir artış, taze sıkılmış elma ve portakal sularındaki PPO ve PME inaktivasyon oranının 10 kat artırmaktadır. Genlik ve basınç artışının taze sıkılmış meyve sularında manosonikasyon (MS) inaktivasyonunun etkisini arttırdığı farkedilmiştir. TT, MS ve MTS uygulamaları karşılaştırıldığında, birlikte uygulanan MTS'in enzim aktivitesinin azaltılmasında daha etkili olduğu bulunmuştur. Elma suyunda bulunan PPO enzimi inaktivasyonu üzerinde MS uygulamasının etkisi, TT ve MTS uygulamalarının etkilerinden daha azdır. PPO düşük ısıl dayanıklılığa sahip bir enzim olarak nitelendirildiğinden, MTS uygulaması sırasında ısıl inaktivasyon daha etkili olmaktadır. Buna karşılık, portakal suyunda bulunan PME enziminin yüksek ısıl dayanıklılığı nedeniyle MS ve MTS inaktivasyonu, ısıl inaktivasyondan daha etkilidir. Bu çalışmaya göre portakal suyundaki PME enziminin ultrases direnci elma suyundaki PPO enziminin direncinden daha fazladır. Uygulama şartlarına bağlı olarak, asidik meyve sularında MTS uygulaması sinerjistik inaktivasyon etkisi göstermekte ve sinerjistik etkinin büyüklüğü iki enzim içinde aynı olarak değelendirilmektedir.

Anahtar Kelimeler: ultrases, manosonikasyon, manotermosonikasyon, *Listeria monocytogenes*, *Escherichia coli* O157:H7, polifenolosidaz, pektinmetilesteraz, elma suyu, portakal suyu.

To my son DEMİR GÜZEL
and
my husband GÖKHAN GÜZEL
''The meanings of my life''

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LIST OF ABBREVIATIONS

US : Ultrasound
MS : Manosonication
TS : Thermosonication
TT : Thermal Treatment
MTS : Manothermosonication
PPO : Polyphenol Oxidase
PME : Pectinmethly Esterase
LOX : Lipoxygenase
POD : Peroxidase
PG : Polygalacturanase
Oxy- DPO : Oxy- Diphenoloxidase
USFDA : US Food and Drug Administration
TSAYE : Tryptone Soy Agar supplemented with 0.6% of yeast extract
TSBYE : Tryptone Soy Broth supplemented with 0.6% of yeast extract
TR-SC : Thermo-resistometer Sala-Condón
CFU : Colony Forming Unit
D : Decimal reduction time
$\boldsymbol{D_{MS}}$: Decimal reduction time for manosonication
$\boldsymbol{D_{MTS}}$: Decimal reduction time for manothermosonication
$\boldsymbol{D_{TT}}$: Decimal reduction time for thermal treatment
$\boldsymbol{z_{TT}}$: °C increase in temperature for D_{TT}-value to drop 1 log cycle
$\boldsymbol{z_{MS}}$: W increase in power for D_{MS}-value to drop 1 log cycle
UHT : Ultra high temperature

CHAPTER 1

INTRODUCTION

Foods begin to lose their quality through changes resulting from physical, chemical, enzymatic, or microbiological effects. The food preservation techniques use to increase the shelf - life and safety of food products.

Raso and Barbosa – Cánovas (2003) explained the characteristics of an ideal method of food preservation techniques:

- It improves shelf life and safety by inactivating spoilage and pathogenic microorganisms.
- It does not change organoleptic and nutritional attributes.
- It does not leave residues.
- It is cheap and convenient to apply.
- It encounters no objections from consumers and legislators.

Today, thermal treatment is the most common processing method for food preservation to inactivate microorganisms and enzymes. Thermal processing supplies most of the characteristics of an ideal food preservation method. However, it can cause undesirable alterations of sensory attributes, such as smell, texture, color, flavor, and nutritional qualities. Consumers demand for fresh-like food, which minimally processed with high sensory and nutritional attributes, therefore non-thermal food processing and preservation methods gain importance. Fruit and vegetable products can be nonthermally processed by ionizing radiation, ultraviolet-C light, high pressure, ultrasound, pulsed electric fields, light pulses and oscillating magnetic fields (Bosiljkov et al., 2010). The use of nonthermal processes in combination with other preservation technologies presents a number of potential benefits to food preservation (Raso and Barbosa - Cánovas, 2003). Nonthermal methods allow the processing of fruit and vegetable products below temperatures used during thermal processing. In addition to improving the shelf life and safety of foods, nonthermal preservation methods or combinations of such processes with mild heat treatment is very attractive for the food industry, because it provides the opportunity to introduce safe products in food market.

Food preservation using combined methods involves successive or simultaneous applications of various individual treatments. Combined treatments are advantageous, principally because many individual treatments applied alone are not adequate to ensure food safety or stability. In some circumstances, combined treatments allow a milder use of single treatments, with a consequent improvement in the food quality. For conventional preservation treatments, optimal microbial control achieved through the hurdle concept, with synergistic effects resulting from different components of the microbial cell being targeted simultaneously (Leistner and Gorris, 1995; Leistner, 2000).

In this investigation, it is interested in ultrasound and their effects on fruits juices. Ultrasound is one of the simplest and most versatile method for the disruption of cells because of it being efficient, safe and reliable (McClements, 1995; Povey, 1998; Kuldiloke, 2002; Coupland, 2004; Kantas, 2007).

Apple and orange juices are some of the most common fruit juices in Turkey and as well in the world. The consumption of noncarbonated fruit juice, both canned and pasteurized ready-to-serve drinks and concentrates, frozen or pasteurized, has become increasingly important in the world. Juices obtained by the extraction of cellular juice from fruits with either pressing or diffusion technique are a product for a direct consumption. Fruit juices are categorized as the one without pulp (‘‘clarified’’ or ‘‘not clarified’’) and with pulp (‘‘pulps,’’ ‘‘pure´es,’’ and ‘‘nectars’’). The juices from a wide variety of fruits such as apples and oranges can extracted to produce natural beverages. The processes used vary considerably depending on the type of fruit, their age and maturity

Apples are climacteric fruit, with increasing respiration as the fruit matures. The ripening stage will continue during post harvesting. The composition of apple juice is dependent on the fruit variety. The minimum and maximum values of °Brix and pH are 9.8 and 16.9, and 3.23 to 6.54, respectively (Keller and Miller, 2005). Apple juice is a fruit juice manufactured by the maceration and pressing of apples. It is a component of several cocktails; it also used as filler in some drinks, because it is less expensive and more widely available than most other juices. It may also produce and consume in a carbonated form, referred to as sparkling apple juice. Moreover, cloudy and clarified apple juices also consume in the world.

Apple juice contains a considerable portion of the soluble constituents of the original apple, as for example, sugars, acids, vitamins, phenols, antioxidants, other carbohydrates and minerals (Iyıdogan, 2000; Podsedek et al., 2000; Baumann et al., 2005). The predominant sugar in apple juice is levulose with small amounts of sucrose and glucose. Malic acid has been the only generally recognized acid present in apple juice. When the acidity of an apple juice referred to, it usually states in terms of malic acid. Tannin is also present in small amounts in apple juice. This group of compounds has a marked effect on the flavor of juice because of their astringency. Tannins are also partially responsible for the rapid darkening of macerated apple tissue and apple juice when they expose to the air (Tressler and Joslyn, 1971; Potter and Hotchkiss, 1998). In the literature, two enzyme systems are responsible for the oxidative browning of ground up apple tissue and juice (Iyidogan, 2000). These are polyphenol oxidase (PPO) and peroxidase (POD). Polyphenol oxidase is responsible for the greater part of the discoloration as it oxidizes the catechol and pyrogallol of the tannins in apple juice. Peroxidase is responsible for only small part of the darkening and it is not required to produce discoloration. *dl*-catechin and chlorogenic acid are tannins involved in phenolase catalyzed oxidations (Tressler and Joslyn, 1971; Potter and Hotchkiss, 1998; Iyidogan, 2000).

Orange juice is a drink derived generally from the citrus fruit *Citrus sinensis* which is sweet orange (Kimball, 1991). It is the most important fruit juice for the beverage processing industry worldwide with a share of approximately 60 % of the total fruit juice trade (Akdag, 2008; Muñoz et al., 2011). Many customers prefer concentrated orange

juice because of an easily storage and transportation. Citrus fruit contain several phytochemicals and/or nutraceuticals including vitamin C that have antioxidant properties and reportedly reduce the risk of cardiovascular diseases and some forms of cancer (Economos and Clay, 1999; Muñoz et al., 2011). The pH of orange juice generally is 3.5, because of the citric acid. Some factors such as variety, growing conditions, maturity, various practices and treatments, climate and, rootstock affect the composition of citrus juice (Tressler and Joslyn, 1971; Kimball, 1991).

Citrus juices are preferred in a cloudy form, which is similar to the natural citrus juices (Kimball, 1991; Economos and Clay, 1999). A critical orange juice quality parameter is cloud stability similar to imparting characteristic color, mouth feel, and flavor to orange juice. Its color is a primary factor considered by the consumer in determining juice quality and correlated with both sensorial and nutritional quality attributes (Tiwari, et al., 2008). The pectinase enzymes are responsible for the cloud stability in the orange juices. These enzymes are also referred as pectinesterases (PE) or pectinmethylesterases (PME) (Kimball, 1991).

1.1 Fruit Enzymes and Related Quality Attributes

Enzymes can play a key role in these processes improving yield, clarity and stability of the juice. Fruit and vegetables also contain natural enzymes. Some key enzymes in fruit and vegetable processing include (Dede, 2005):

- polyphenoloxidase (PPO) which is responsible for enzymatic browning
- pectinmethylesterase (PME) which is responsible for cloud destabilization and consistency changes
- lipoxygenase (LOX) which induces changes in flavor, color and nutritional value
- peroxidase (POD) which gives rise to unfavorable flavors.

Processing including trimming, peeling, cutting, and other physical actions such as pasteurization, sterilization and blanching can alter and often damage fruit and vegetable functional components. Any processing method that maintains the level of compounds known for their health benefits will be of interest to the food industries (Economos and Clay, 1999). Inactivation of many enzymes generally uses as an indicator of the adequacy of pasteurization because it is known to be more heat resistant than the common microorganisms. In our study, we are interested in the well-known heat resistant enzymes such as PPO in apple and PME in orange.

1.1.1 Polyphenoloxidase

As a member of the oxidoreductase group, PPO (EC 1.10.3.1- *o*-diphenol; catecholase) is a copper containing enzyme catalyzing the hydroxylation of monophenols to *o*-diphenols (creasolase or monophenolase activity) and oxidation of *o*-diphenols to their *o*-quinones (catecholase or diphenolase activity) of phenolic compounds in fruits and vegetables. During this reaction, PPO spontaneously polymerize into red, brown or black pigment (melanin) in the presence of molecular oxygen (Mayer and Harel, 1979; Mayer, 1987;

Janovitz-Klapp et al., 1990; Mayer and Harel, 1991; Tchoné et al., 2005; Buckow et al., 2009). Polyphenoloxidase is named as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase and catecholase at various times. The nomenclature of these enzymes is somewhat confusing because the three numbers exist (EC 1.10.3.1, EC 1.10.3.2 and EC 1.14.18.1) (Iyidogan, 2000). The three types of proteins related to PPOs are catechol oxidase (EC 1.10.3.1), laccase (EC 1.10.3.2) and cresolase (EC 1.14.18.1) (Sheptovitsky and Brudvig, 1996; Iyidogan, 2000; Krebs et al., 2004; Tchoné et al., 2005). Because of this latter property, PPO is an important enzyme in the food industry (Tchoné et al., 2005).

Many authors recognized that PPO is a main source of enzymatic browning (Whitaker et al., 1995; Sheptovitsky and Brudvig, 1996; Podsedek et al., 2000; Tchoné et al., 2005). Formation of brown pigments occurs, when tissue of plant damaged by slicing, cutting or pulping. Such brown pigments are undesirable with respect to color or flavor and finally nutritional quality of the fruit products are affected negatively (Janovitz-Klapp et al., 1990; Espin et al., 1995a, 1995b; Oktay et al., 1995; Yemenicioğlu et al., 1997; Hendrickx et al., 1998; Lee et al., 2009a). PPO activity, polyphenolic components and oxygen are factors influencing the rate of enzymatic browning in apples (Coseteng and Lee, 1987; Murata et al., 1995; Hendrickx, et al., 1998; Podsedek et al., 2000). PPO catalyzing the oxidation of polyphenols to corresponding quinones is a key enzyme of enzymatic browning (Tchoné et al., 2005). An enzyme of the oxidoreductase class catalyzing the reaction between catechol and oxygen to yield benzoquinone and water can be seen in the Figure 1.1.

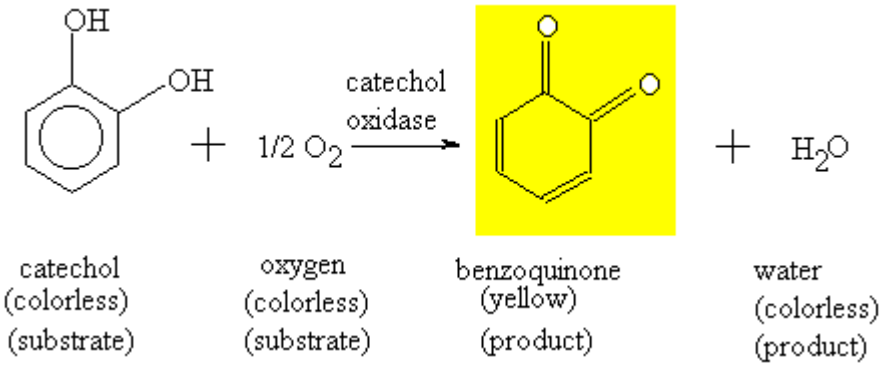

Figure 1.1. Reaction catalyzed by PPO (Krebs et al., 2004)

PPO catalyzes enzymatic browning and creates melanins. The physical damage to the plant tissue in which naturally occurring monophenolic compounds mixed with atmospheric oxygen and endogenous polyphenol oxidase enzymes are hydroxilated to *o*-diphenols. The oxidation of *o*-dihydroxy phenols by PPO occurs, most probably, according to an ordered sequential mechanism. Since PPO contains copper as the prosthetic group and for the enzyme to act on its substrate phenols the Cu^{+2} must first

reduced to Cu^{+} in which state the enzyme can bind O_2. The phenolic substrates bind only to the *oxy*-diphenoloxidase (*oxy*-DPO) part and, because of this binding, hydroxylation of the monophenol or oxidation of the diphenol occurs. *O*-diphenols and together with endogenous *o*-diphenols are then oxidized to *o*-quinones (Figure 1.1). These quinones may condense and react non-enzymatically with other phenolic compounds, amino acids, proteins, and other cellular constituents to produce colored polymers and pigments. The reaction resulting in the formation of *o*-quinones is reversible while subsequent reactions are not. *o*-quinones can react with phenols, resulting in molecules of phenols. These molecules, having an *o*-diphenolic structure, subject to reoxidation either enzymatically or by other *o*-quinones, resulting in the formation of larger oligomers with different color intensities. The *o*-quinones can further react with phenols, either leading to copolymers or regeneration of these phenols giving different o-quinones by coupled oxidation. Browning reactions can also occur with nonphenolic compounds such as ascorbic acid, sulfites and amino acids. Coupled oxidation with ascorbic acid regenerates phenols resulting in the formation of dehydroascorbic acid. With sulfites, colorless addition compounds formed together with regenerated phenols. The *o*-quinones also form addition compounds with thiol groups by nucleophilic additions. Cysteine, either free or bound in small peptides or large proteins, gives colorless compounds. Although these compounds have an *o*-diphenolic structure, they are not a substrate of PPO but can either be oxidized by laccase or react with an excess of *o*-quinones by a coupled oxidation mechanism and form intensely colored products (Nicolas et al., 1994; Iyidogan, 2000; Krebs, et al., 2004).

Enzymatic browning cause severe quality defects during handling of light-colored fruits and vegetables (López et al., 1994). It generally considered to an undesirable reaction due to the unpleasant appearance and development of an off-flavor. It creates heavy economic losses for producers (Sanchez-Amat and Solano, 1997). This reaction is important in food preservation and processing and its regulation is important in the food industry (Tchoné et al., 2005).

1.1.2 Pectinmethylesterase

The term pectin refers to a class of high molecular weight compounds, with molecular weights of 100000 to 200000, consisting of 150 to 1500 galacturonic acid units linked together via α (1→ 4) glycoside bonds with side chains of rhamnose, arabinans, galactans, xylose, and fucose (Kimball, 1991) (Figure 1.2). Pectin esterase (EC 3.1.1.11) is a ubiquitous cell wall associated enzyme that presents several iso-forms that facilitate plant cell wall modification and subsequent breakdown. It is found in all higher plants as well as in some bacteria and fungi. Pectin esterase functions primarily, by altering the localized pH of the cell wall resulting in alterations in cell wall integrity (Prasanna et al., 2007).

PME is responsible for cloud destabilization of juices (orange), gelation of concentrates and consistency loss of products (Hendrickx et al., 1998). PME catalyzes the deesterification of pectin molecules, which may then form insoluble complexes with calcium ions, leading to cloud loss, phase separation in single-strength orange juices and gelation in their concentrates (Kimball, 1991; Pilnik and Voragen, 1991; Basak and

Ramaswamy, 1996; Vercet et al., 1999; Dede, 2005; Yıldız and Baysal, 2006). This degradation of the pectin chains also reduces the viscosity of the juice and diminishes the quality of juice products (Yıldız and Baysal, 2006; Wu et al., 2008).

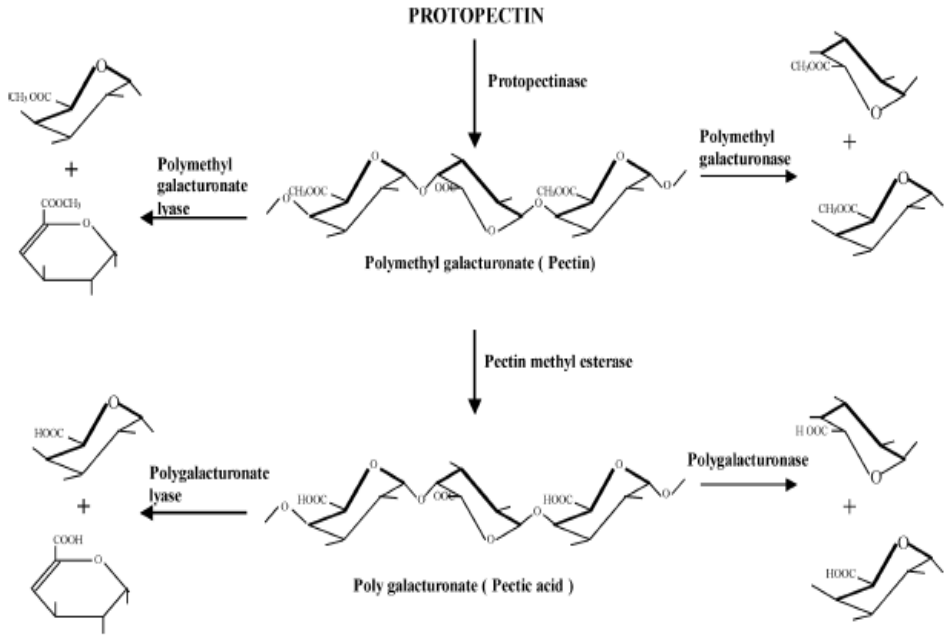

Figure 1.2: Mode of action of pectin degrading enzymes (Prasanna et al., 2007)

Two basic types of reactions result in a gelation in citrus concentrates or cloud loss in single-strength citrus juices. One reaction is reversible acid- or base-catalyzed esterification of the carboxyl groups of the galacturonic acid components of pectins. The equilibrium of the reaction depends on many factors, including pH, water content, heat, and secondary reactions. In the presence of sugars, under the proper conditions, polymerization can occur in citrus concentrates, resulting in gelation. Even though the methoxyl groups them, prevent such polymerization that is not common in citrus concentrates. Another type of deesterification, which can lead to cloud loss and/or gelation trough calcium-pectate formation, results from the pectinase enzymes that naturally occur in citrus juices (Kimball, 1991).

The PME is accepted as the indicator enzyme in heat inactivation studies because it is the most heat resistant pectolytic enzyme (Versteeg et al., 1980; Van Den Broeck et al., 2000; Yıldız and Baysal, 2006). In addition, PME from different fruits has been reported to be quite thermo resistant: temperatures between 80 and 95 °C are required to induce significant inactivation and even then PME remains active (Van Den Broeck, 2000). This resistance can be explained the presence of heat labile PME iso-enzymes (Versteeg et al., 1980; Van Den Broeck et al., 2000). Stabilization of the cloud in citrus juices requires the inactivation or inhibition of PME (Eagerman and Rouse, 1976; Kimball, 1991; Vercet et al., 1999).

1.2 Fruit Microorganisms and Related Quality Attributes

Historically, acid foods such as fruit juices have been considered safe, however, recent food borne disease outbreaks attributed to unpasteurized juices contaminated with pathogens. Such as *Salmonella, Cryptosporidium parvum, Listeria monocytogenes* and *Escherichia coli* O157:H7 have demonstrated that unpasteurized juice can be a vehicle for food-borne illness. Since these and other acid-tolerant pathogens may be able to grow in the juice and cause infections at low doses, there could be a need to include pathogen control measures during a juice manufacture. To produce a juice with no danger of spoilage a process of 5.5 log microbial reduction is needed (Tressler and Joslyn, 1971; USFDA, 2001; Ugarte-Romero et al., 2007; Adekunte et al., 2010a, 2010c).

Yeasts such as *Saccharomyces cerevisiae, Zygosaccharomyces bailii*, and *Zygosaccharomyces rouxii* and pathogenic bacteria like *L. monocytogenes* and *E. coli* can cause significant spoilage and affect the safety of non-pasteurized fruit juice products (Keller and Miller, 2005). Bacteria are frequently found in fruit and vegetable juices. The so-called lactic acid, acetic acid and butyric acid bacteria deserve special consideration for spoilage, because they are related to food poisoning.

Natural micro-flora found in fresh apple juice also changes with apple variety and significantly affects by pH, percentage titratable acidity, and °Brix. Total aerobic microbial populations in freshly squeezed juice from tree-harvested fruits were measured from 1.90 to 3.40 log CFU/mL. Yeast and mold populations in the same juice also changed from 1.99 to 3.32 log CFU/mL. Total aerobic microbial, yeast and mold population measure in poorer quality harvest fruit. For this group, juice microbial populations are substantially higher, ranging from 4.19 to 5.43 log CFU/ml for total aerobic populations and from 3.84 to 5.23 log CFU/ml for yeast and mold populations. Typically, aerobic microbial load on citrus fruit is approximately 4.0 log CFU/cm^2. Yeast and mold population measure alone seem to show greater variability than total aerobic populations, but reported as nearly as high total aerobic microbial load. In citrus juices, acidic conditions, coupled with higher sugar content, result in a microbiological population made up primarily of acidolactic bacteria, yeasts and molds (Kimball, 1991; Keller and Miller, 2005).

1.2.1 *Listeria monocytogenes*

Listeria monocytogenes is a gram positive, rod-shaped, facultative anaerob, non-spore forming bacteria, in the division *Firmicutes*, named for Joseph Lister. Motile via flagella at 30°C and below but usually not at 37°C, *L. monocytogenes* can instead move within eukaryotic cells by explosive polymerization of actin filaments (Faber and Peterkin, 2000; Baumann et al., 2005; Ugarte-Romero et al., 2007). E.G.D. Murray firstly described *L. monocytogenes* in 1924 based on six cases of sudden death in young rabbits. Murray et al. (1926), referred to the organism as *Bacterium monocytogenes* before Pirie (1940), changed the genus name to *Listeri*a. Although clinical descriptions of *L. monocytogenes* infection in both animals and humans were published in the 1920s, it was not recognized as a significant cause of neonatal sepsis and meningitis until 1952 in East

Germany (Hof, 2003). *L. monocytogenes* was identified as a cause of food borne illness after 80s (Schlech et al., 1983). Since then a number of cases of food-borne diseases has been reported, and *L. monocytogenes* is now widely recognized as an important hazard in the food industry (Hof, 2003).

Infection by *L. monocytogenes* causes the disease listeriosis. The manifestations of listeriosis include fever, nausea, vomiting, diarrhea, septicemia (blood-borne), meningitis (or meningoencephalitis), encephalitis, corneal ulcer, pneumonia, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2nd/3rd trimester) or stillbirth. The onset time to serious forms of listeriosis is unknown but may range from a few days to three weeks. In addition, the onset time to gastrointestinal symptoms is unknown but probably exceeds 12 hours. An early study suggested that *L. monocytogenes* was unique among gram-positive bacteria in that it possessed lipopolysaccharide, which served as an endotoxin. The infective dose of *L. monocytogenes* varies with the strain and with the susceptibility of the victim. The pathogenesis of *L. monocytogenes* centers on its ability to survive and multiply in phagocytic host cells (Altekruse et al., 1997; Hof, 2003; Keller and Miller, 2005; Ugarte-Romero et al., 2007).

The ubiquitous presence of *L. monocytogenes* makes it possible to contaminate nearly all kind of foods (Macgowan et al., 1994; Hof, 2003; Ugarte-Romero et al., 2007). *L. monocytogenes* has also been isolated from unpasteurized apple juice (Bauman, et al., 2005; Keller and Miller, 2005). The minimum pH for growth of *L. monocytogenes* is dependent on the acidulant. For malic acid, the primary acid found in apple juice, the lowest pH value for growth of *L. monocytogenes* is from 4.4 to 4.6 depending on the strain (Yuste and Fung, 2002; Keller and Miller, 2005; Ugarte-Romero et al.,2007). In addition, although *L. monocytogenes* may not grow at lower pH values, survival at lower pH similar to *E. coli* O157:H7 and *Salmonella* is possible. The recently completed *L. monocytogenes* risk assessment indicated that consumption of fresh fruit has a low risk for listeriosis. However, two risk factors need to consider concerning juice-associated listeriosis. First, commingling fruit to make juice spreads the risk over a much larger exposed population, when compared to a single or limited serving size typically associated with the fruit itself. Second, fresh juice is frequently consumed by subpopulations at risk for listeriosis. Consequently, it is reasonable to consider as somewhat likely outbreaks or sporadic cases of listeriosis associated with fresh juices (Keller and Miller, 2005).

1.2.2 *Escherichia coli* O157:H7

Escherichia coli is a gram negative, facultative anaerobic and non-sporulating bacterium that is commonly found in the lower intestine of warm-blooded animals (Erkmen & Bozoglu, 2008). Cells are typically rod-shaped, and are about 2.0 micrometers (μm) long and 0.5 μm in diameter, with a cell volume of 0.6 – 0.7 $(\mu m)^3$. Some strains that possess flagella can swim and are motile. The flagella have a peritrichous arrangement. Most *E. coli* strains are harmless, but some, such as serotype O157:H7 can cause serious food poisoning in humans. Because of an outbreak of unusual gastrointestinal illness in 1982, *E. coli* O157:H7 was firstly recognized as a pathogen. The etiologic agent of the illness

was identified as a rare O157:H7 serotype of *E. coli* in 1983. This serotype had only been isolated once before from a sick patient in 1975. The letter "O" (not a zero) in the name refers to the somatic antigen number, whereas the "H" refers to the flagella antigen. While most strains are harmless and normally found in the intestines of mammals, this strain may produce Shiga-like toxins, cause severe illness, and is a member of a class of pathogenic *E. coli* known as enterohemorrhagic *Escherichia coli* or EHEC (Keller and Miller, 2005; Erkmen and Bozoglu, 2008).

E. coli O157:H7 is markedly different from other pathogenic *E. coli*, as well. It does not produce heat-stable or heat-labile toxins and is non-hemolytic. *E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process led to the spread of the gene encoding shiga toxin from *Shigella* to *E. coli* O157:H7, carried by a bacteriophage. *E. coli* O157:H7 serotypes apparently arose because of horizontal gene transfer of virulence factors. Among these virulence factors are a periplasmic catalase and shiga-like toxins. Shiga-like toxins are iron-regulated toxins that catalytically inactivate 60S ribosomal subunits of eukaryotic cells blocking mRNA translation and causing cell death. Shiga-like toxins are functionally identical to toxins produced by virulent *Shigella* species. Strains of *E. coli* that express shiga-like toxins gained this ability due to infection with a prophage containing the structural coding for the toxin, and non-producing strains may become infected and produce shiga-like toxins after incubation with shiga toxin positive strains (Keller and Miller, 2005; Erkmen and Bozoglu, 2008).

E. coli O157:H7 infection often causes severe, acute bloody diarrhea (although non-bloody diarrhea is also possible) and abdominal cramps. Usually little or no fever is present, and the illness resolves in 5 to 10 days. It can also be asymptomatic and cause hemolytic-uremic syndrome and sudden kidney failure (Keller and Miller, 2005; Erkmen and Bozoglu, 2008; Feng, 2012).

E. coli serotypes O157:H7 are not normal endogenous micro flora of fresh juice or of the fruit used to produce fresh juice. Their presence on fruit and in fruit juice is believed to be the consequence of some form of fecal contamination prior to consumption. From epidemiological data, it is clear that *E. coli* O157:H7 can survive well enough in low pH juice to result in serious illness. Although the pH of most apple and orange juice is low enough to either significantly slow or inhibit growth of *E. coli*, these strains have tolerance to high acid levels of acid allowing for extended survival time. Tolerance to high acid levels is a complex induced response involving three distinct mechanisms and enhanced in stationary phase cells (Keller and Miller, 2005).

1.3 Sound

Sound consists of mechanical vibration of the molecules or atoms of a solid, liquid, or gas material about the equilibrium positions of these particles; particularly, sound means those mechanical vibrations composed of frequencies capable of being detect by human ears (Goldman, 1962; Suslick, 1988; Gonzalez, 2003).

Low and high frequency vibrations in air cannot be heard by human (<16 kHz). These vibrations travel through all forms of matter, solids, liquids, gases, and plasmas, also called medium. A movement of mechanical energy through medium that causes a change on the surrounding pressure is commonly definitions of sound to use in the scientific and engineering areas (Gonzalez, 2003).

Sound is an invisible vibration. It travels in waves, spreading outwards from the source of the sound. In other words, these invisible vibrations are carried by the medium in the form of "waves" (Glickstein, 1960; Goldman, 1962; Blitz, 1971; Henderson, 2013). When a wave disturbance is produced into the matter, the particles begin to vibrate back and forward about an average position at the same speed (Figure 1.3). At any given moment in time, a particle on the medium could be above or below the rest position. The crest of a wave is the point on the medium, which exhibits the maximum amount of positive or upper displacement from the rest position. A second crest that is often followed by a third crest often follows one crest. In Figure 1.3, points A, E and H are crest points of wave. The trough of a wave is the point on the medium, which exhibits the maximum amount of negative or lower displacement from the rest position. A second trough that is often followed by a third trough often follows one trough. In Figure 1.3, points C and J are trough points of wave. Points D, G and I are the equilibrium or rest points of particles (Henderson, 2013).

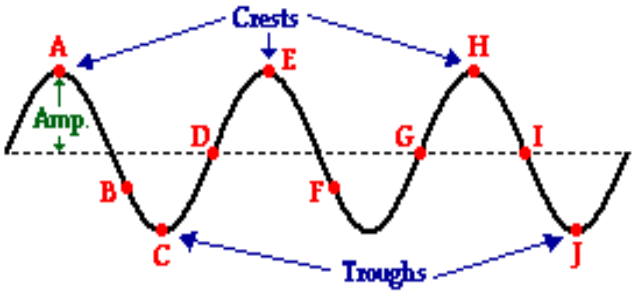

Figure 1.3. Crests and troughs (Henderson, 2013)

Sound waves include compression and rarefaction areas that follow each other. The upwards and downwards motion of particles in a medium accounts for the alternate rarefaction and compression portions of a wave. The area of high pressure is named by compression and the area of low pressure is named by rarefaction (Figure 1.4) (Glickstein, 1960; Goldman, 1962; Blitz, 1971; Henderson, 2013).

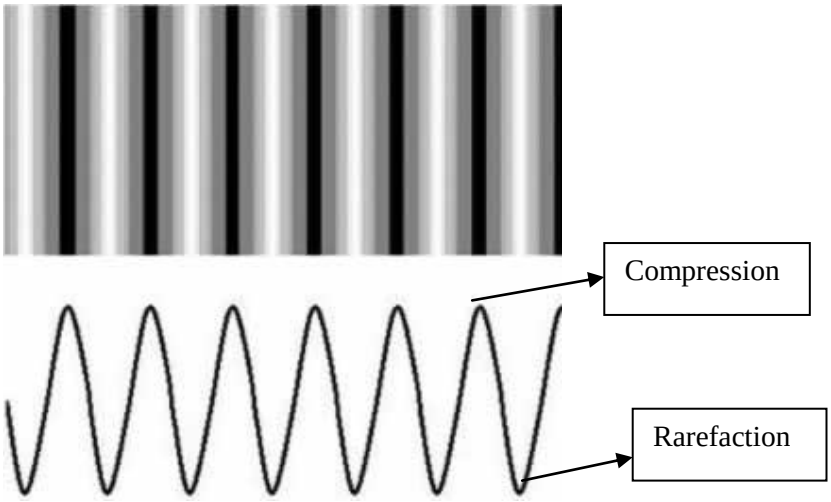

Figure 1.4. Motion of sound in a medium (Henderson, 2013)

When sound propagates into the medium, three things affect the behavior of it: (Henderson, 2013)

a) A relationship between density and pressure
b) The motion of the medium
c) The viscosity of the medium

Sound is divided into three main areas: (Figure 1.5)

1) Infrasound (below audible sound or < 16 Hz),
2) Audible sound (16 Hz-20 kHz) and
3) Ultrasound with too high frequencies (20 kHz- 10 MHz)

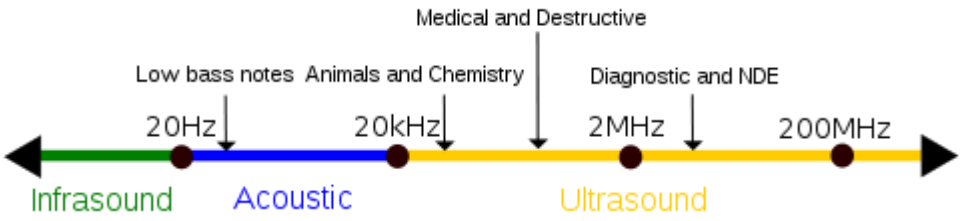

Figure 1.5. Sound range diagram

1.3.1 Waves and Sound Wave

A wave can be described as disturbance travelling through a medium by which energy is transferred from one particle of the medium to another without causing any permanent displacement of the medium itself (Gonzalez, 2003; Kantaş, 2007). A sine and cosine

waves are periodic functions and the simplest forms of this motion (Figure 1.6) (Henderson, 2013)

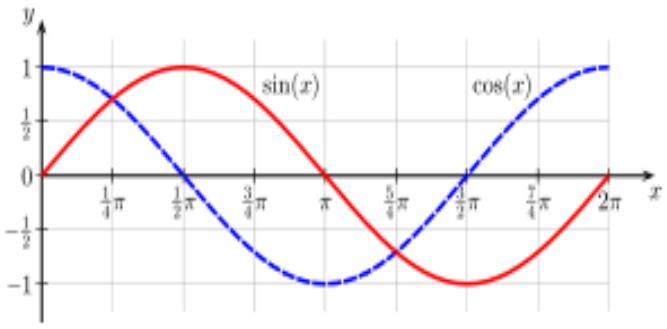

Figure 1.6. A sine and cosine waves are periodic functions (Henderson, 2013)

The sound source that causes a vibration produces the waves. In the surrounding medium, these vibrations disperse and particles of medium disturb each other. This process repeats until all the energy is propagated (Henderson, 2013).

One way to categorize waves is based on their ability or inability to transmit energy through a vacuum (i.e. empty space). Categorizing waves on this basis leads to two notable categories: mechanical waves and electromagnetic waves. The vibration of charged particles produce electromagnetic waves (radio waves, infrared, visible light, ultraviolet, *x*-rays, gamma rays) that are capable of transmitting their energy through a vacuum but this transmission is poor. In addition, they cannot pass through at all most solids and liquids. On the other hand, mechanical waves are not capable of transmitting their energy through a vacuum. They require a medium in order to transport their energy from one location to another. The most common medium is air but solids and liquids can also transmit them. Sound wave is an example of mechanical waves (Glickstein, 1960; Kantas, 2007; Henderson, 2013).

Another way to categorize waves is based on the travel direction of waves. This category has three notable subtitles: transverse waves, longitudinal waves, and surface waves (Glickstein, 1960; Goldman, 1962; Henderson, 2013). In a transverse wave, the particle vibration is different from the wave transmission. This means that when the wave motion transmitted horizontally from one location to the other, the particle vibration is not in the same direction of wave propagation. The transporting direction of energy does not affect by the difference of direction between the particle vibration and the wave motion. Energy will begin to transport from left to right or from right to left. Transverse waves require a relatively rigid medium in order to transmit their energy (Glickstein, 1960; Goldman, 1962; Henderson, 2013). This wave motion can be illustrated in Figure 1.7.

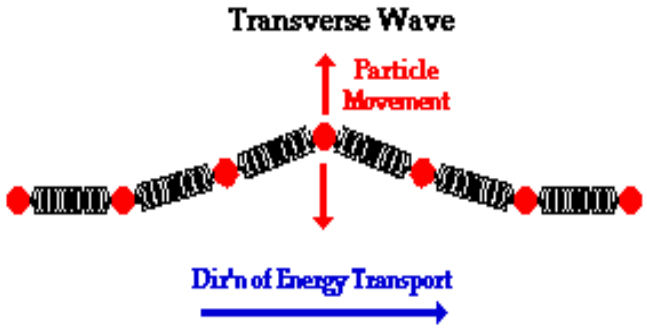

Figure 1.7: Transverse wave motion (Henderson, 2013)

In a longitudinal wave, the particle vibration is in the same direction with the wave transmission. In other words, when the wave motion transmitted horizontally from one location to the other, the particle vibration is in the same direction of sound propagation. The transporting direction of energy is also not affected the directions of particle and wave. Longitudinal waves transmit their energy in solids, liquids, gases and plasmas (Goldman, 1962; Henderson, 2013). This wave motion can be illustrated in Figure 1.8. Sound wave is an example of longitudinal wave. That means the sound wave, particle, and the medium have same direction (Goldman, 1962).

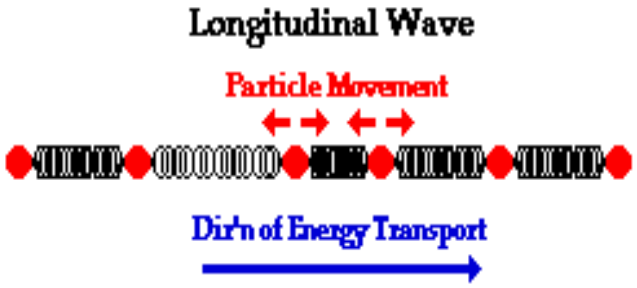

Figure 1.8: Longitudinal wave motion (Henderson, 2013)

A surface wave is different from the longitudinal and transverse waves. In a surface wave, the particles of the medium have parallel, perpendicular and circular motions in surface of the medium. On the other hand, in longitudinal and transverse waves, all particles of the medium move in a parallel and a perpendicular direction respectively. The motion of particles tends to decrease as one proceeds further from the source of the wave (Glickstein, 1960; Henderson, 2013).

1.3.2 Properties of Sound Wave

Sound waves are characterized by the generic properties of waves, which are cycle, period, frequency, wavelength, amplitude, sound intensity, sound pressure, wave number, speed of sound and direction (sometimes speed and direction are combined as a velocity vector, or wavelength and direction are combined as a wave vector).

Cycle: A complete vibration is called a cycle. In other words, at the same speed, back and forward or upwards and downwards motions of the particles in the medium generate a complete vibration, which is a cycle. For example, a sine wave starts from zero point to positive direction, it reaches maximum amplitude (crest point), and it returns the zero point (equilibrium or rest point),then it moves from zero point to negative direction, reaches minimum amplitude (trough point) and again returns the zero point. One cycle is equal to one complete rotation (Kantas, 2007).

Period (T): Period is the time for a particle on a medium to make one complete vibrational cycle. It refers to the time for repeat all complete cycle. It is measured as the units of time such as second, hours, days or years (Henderson, 2013).

Frequency (*f*): The frequency refers to how often the particles of the medium vibrate when a wave passes through the medium. It is measured as the number of complete back-and-forth vibrations of a particle of the medium per unit of time. A common unit of frequency is "Hertz" that is equal to cycle per second.

1 Hertz = 1 cycle / second (1.1)

As a sound wave moves through a medium, the first particle of the medium begins vibrating, and begins to set the second particle into vibrational motion at the same frequency. The second particle begins vibrating and thus sets the third particle of the medium into vibrational motion. The process continues throughout the medium; each particle vibrates at the same frequency. Finally each particle vibrates is the same as the frequency of the original source of the sound wave (Diamantini, 2003; Henderson, 2013).

There is a relationship between period and frequency but they are different quantities. Frequency refers to how often something happens. Period refers to the time it takes something to happen. Frequency is a rate quantity. Period is a time quantity. Frequency is the cycles/second. Period is the seconds/cycle. The symbol f is used for frequency and the symbol T is used for period; these equations are also expressed as (Henderson, 2013):

Period (T) = 1 / Frequency (f) Frequency (f) = 1/ Period (T) (1.2)

$T = 1 / f$ $f = 1 / T$

The second relationship between them is explained with Figure 1.9 that shows two pressure-time plots, one corresponding to a high frequency and the other to a low frequency. When a sound wave is produced with a high frequency, period of wave will be small in the pressure versus time plot. If a sound wave is produced with a low frequency, the period of wave will be large in the pressure versus time plot (Henderson, 2013).

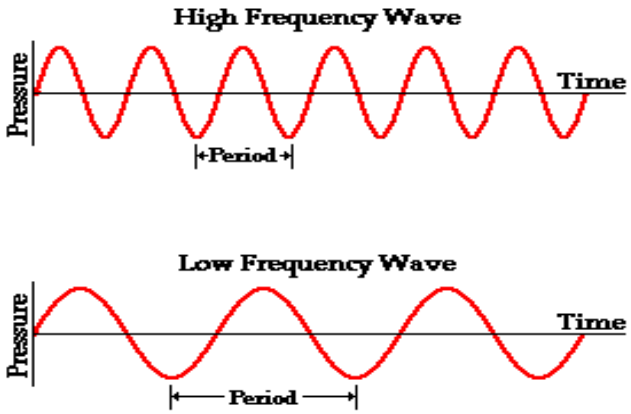

Figure 1.9: Pressure vs. time plot for waves with high and low frequency (Henderson, 2013)

Wavelength (λ) : Wavelength is defined as the distance occupied by one complete cycle (Henderson, 2013). The distance between two successive crests and troughs is another explanation of wavelength (Figure 1.10) (Krasilnikov, 1963).

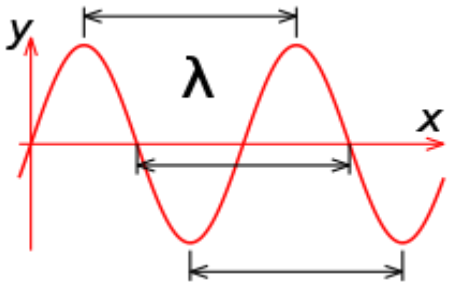

Figure 1.10. Wavelenght of sound (Henderson, 2013)

Amplitude: The amplitude of a wave refers to the maximum amount of displacement of a particle on the medium from its rest position (Henderson, 2013). It is the objective measurement of the degree of change (positive or negative) in atmospheric pressure (the compression and rarefaction) caused by sound waves (Hass, 2003). The amplitude can be measured from rest point to crest or from rest point to trough (Figure 1.11). Amplitude is measured for force applied over an area and the most common unit is the Newtons per square meter (N/m2) (Hass, 2003).

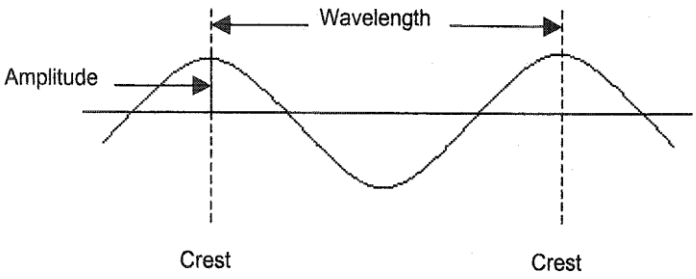

Figure 1.11. Amplitude and wavelength (Gonzalez, 2003)

According to the height of displacement from rest position or from maximum height position, amplitude is divided into four different types (Hass, 2003):

a) Peak amplitude: It is defined as displacement from rest position to crest position or from rest position to trough position (line 1 in Figure 1.12). In scientific, generally it is defined as amplitude.

b) Peak to peak amplitude: It refers to a displacement from crest position to trough position (line 2 in Figure 1.12)

c) Semi amplitude: It means half the peak-to-peak amplitude. Generally, it is used in astronomy and some scientists use amplitude or peak amplitude to mean semi amplitude (line 1 in Figure 1.12).

d) Root mean square (RSM) amplitude: It is defined as the square root of the mean over time of the square of the vertical distance of the graph from the rest state. It is used in electrical engineering (line 3 in Figure 1.12).

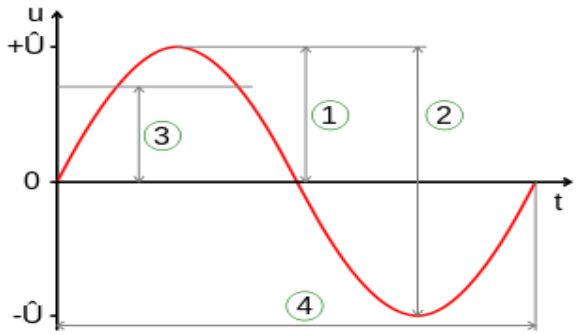

Figure 1.12. Amplitude types 1) Peak amplitude or semi amplitude, 2) Peak to peak amplitude, 3) RSM amplitude, 4) Wavelength of wave.

The amount of energy carried by a wave is related to the amplitude of the wave. A high-energy wave is characterized by high amplitude; a low energy wave is characterized by low amplitude (Figure 1.13). The energy transported by a wave is directly proportional to the square of the amplitude of the wave (Henderson, 2013).

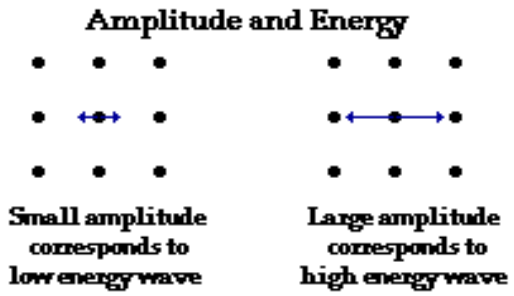

Figure 1.13: Amplitude and energy relationship (Henderson, 2013)

Sound speed (v): The speed of an object is that how fast an object is moving and is usually expressed as the distance travelled per time of travel (Henderson, 2013). In the case of a wave, the speed of a sound wave refers to how fast the disturbance or wave is passed from particle to particle. Since the speed of a wave is defined as the distance, which a point on a wave travels per unit of time, it is often expressed in units of meters/seconds (m/s) (Diamantini, 2003). In equation form,

$$\text{Speed} = \text{Distance} / \text{Time} \quad (1.3)$$

The speed of any wave depends upon the properties of the medium through which the wave is traveling. The density of the medium will affect the speed of the wave, which will travel. A sound wave will travel faster in a less dense material rather than in a more dense material (Diamantini, 2003).

Speed of sound (v) also is related to wavelength (λ) and frequency (f) of sound with mathematically.

$$\text{Speed} = \text{Wavelength} * \text{Frequency} \quad (1.4)$$
$$v = \lambda * f$$

Wavelength and frequency are inversely correlated each other but they are directly proportional with speed.

Sound intensity (I-acoustic energy): The amount of energy that is transported past a given area of the medium per unit of time is known as the intensity of the sound wave.

The term intensity is used exclusively for the measurement of sound in watts per unit area (Henderson, 2013).

Intensity = Energy / (Area * Time) (1.5)
$I = E / (A * t)$

It equals the square of the amplitude, so if the amplitude of a sound is doubled, its intensity is quadrupled. It can be seen as amplitude over time over an area. The intensity is also the product of the sound pressure and the particle velocity (Henderson, 2013)

Intensity = Sound Pressure *Speed (1.6)
$I = P * \nu$

Both amplitude and intensity are relevant to power of sound. The sound power or acoustic power is the energy rate that is the energy of sound per unit of time (J/s, W in SI-units) from a sound source (Henderson, 2013).

Power = Intensity * Area (1.7)
$P = I * A$

The amount of acoustic power entering the medium is very important to determine the some quantities with related to ultrasonic. To measuring the sound power, four categories exist in the literature (Berlan and Mason, 1991; Raso et al., 1999; Löning et al., 2002; Gonzalez, 2003; Kobus and Kusińska, 2008):

1) Thermal methods
    a. Calorimeters: the measurement of temperature increase
    b. Acoustic dilatometer: measuring the rate of thermal expansion of a liquid in a tube
    c. Thermal probes: the direct use of thermal probes
2) Measurement methods with transducers
    a. Electrical impedance: not reliable
    b. Mechanical measurement at the transducer: for solids
    c. Amplitude displacement: the measurement of the amplitude at the tip of the horn
3) Methods based on direct mechanical effects
    a. Acoustical probes: microphones, hydrophones, etc
    b. Optical methods
    c. Acoustic impedance
4) Methods based on secondary effects of sound propagation
    a. Acoustic output and noise measurement
    b. Sonoluminescence

A sound wave carries its energy through a two-dimensional or three-dimensional medium. The intensity of the sound wave decreases with increasing distance from the source. The decrease in intensity with increasing distance is explained by the fact that the wave is spreading out over a circular (2 dimensions) or spherical (3 dimensions) surface

and thus the energy of the sound wave is being distributed over a greater surface area (Krasilnikov, 1963, Hass, 2003; Henderson, 2013)

Sound pressure (P): Sound pressure or acoustic pressure is the local pressure deviation from the ambient (average, or equilibrium) atmospheric pressure caused by a sound wave. Sound pressure can be measured using a microphone in air and a hydrophone in water. The SI unit for sound pressure is the pascal (Pa) (Henderson, 2013).

For small amplitudes, sound pressure and particle velocity are linearly related and their ratio is the acoustic impedance. The acoustic impedance depends on both the characteristics of the wave and the transmission medium. The acoustic impedance is given (Henderson, 2013):

Acoustic impedance (Pa·s/m) = Sound pressure (Pa) * Velocity (speed- m/s) (1.8)

$Z = P * \nu$

Wave number: The wave number is proportional to the reciprocal of the wavelength. It can be defined as the number of wavelengths per unit distance, that is, $1/\lambda$ where λ is the wavelength (Henderson, 2013).

Direction: It is the information contained in the relative position of one point with respect to another point without the distance information (Henderson, 2013).

1.3.3. Transmission of Sound Wave in Liquids

According to acoustical point, solids and liquids are different from gas medium. They are much denser and less compressible than gas. When the acoustic pressure changes very small amount, they slightly compressed. Because of these small changes, sound waves can propagate in these media (Kantas, 2007).

Sound waves are longitudinal waves. The propagation of waves in liquids is only possible for longitudinal waves. They can be generated in liquids, because the energy travels through the atomic structure by a series of compressions and rarefaction movements.

The transmission of sound wave is also related to speed of sound that depends on the density of medium and modulus of elasticity. When a sound wave passes through a fluid, there is a small alternating increase and decrease in static pressure. All these processes cause the alternations in density and temperature. When the temperature remains constant, the isothermal elastic modulus can be measured. However, sound passes through the liquid, during the compression phase of a cycle, the temperature increases, and during the rarefaction phase, it falls. These temperature changes are named by adiabatic and the measured elastic modulus is adiabatic elastic modulus. For liquids, the difference between isothermal and adiabatic elastic modulus can usually be neglected (Hueter and Bolt, 1960; Krasilnikov, 1963; Biltz, 1971).

1.4 Ultrasound

Ultrasound is acoustic energy generated by sound waves with frequencies above the human hearing range. The highest frequency that the human ear can detect is approximately 20 kHz, but some scientific sources accept that higher than 16 kHz frequency is ultrasound. At this point, the sonic range ends and the ultrasonic range begin. Ultrasound is used in many areas such as electronic, navigational, industrial, security and medicine.

The broad classification of ultrasound as sound above 20 kHz and up to 100 MHz can be subdivided into two distinct regions (Mason et al., 1996; Kuldiloke, 2002) (Figure 1.14)

a) Power ultrasound: ultrasound with above 20 kHz frequency
b) Diagnostic ultrasound: ultrasound with above 100 MHz frequency

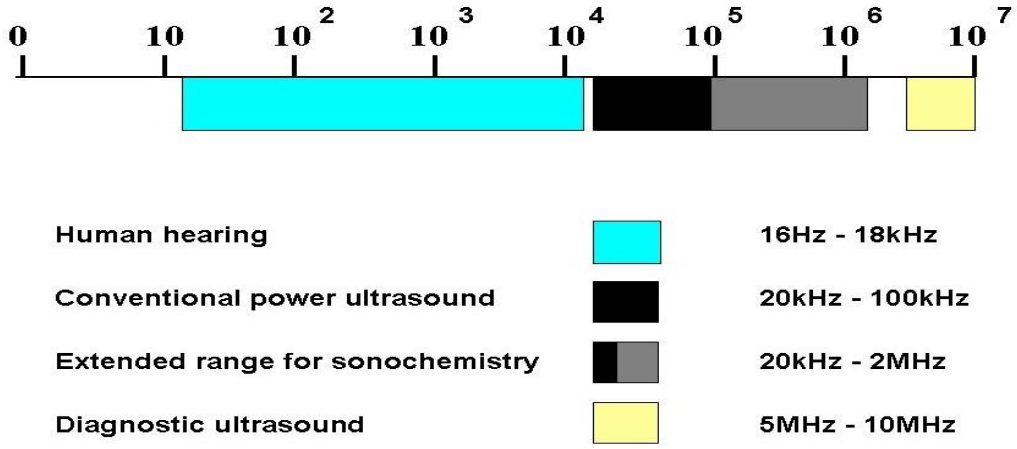

Figure 1.14. The frequency range of sound (Mason, 2012)

Power ultrasound can be divided three main regions:

a) Low frequency, high power ultrasound (20-100 kHz)
b) High frequency, medium power ultrasound (100 kHz – 1 MHz)
c) High frequency, low power ultrasound (1- 10 MHz) (Raso et al., 1999; Joyce et al., 2003; Patist and Bates, 2008; Mason, 2012)

The upper limit of ultrasound is not sharply defined but it is usually taken to be 5 MHz in gases and 500 MHz in liquids and solids (Kuldiloke, 2002).

1.4.1 Effects of Ultrasound

Sound has a number of effects that are physical, chemical and biochemical. The high power ultrasonic energy produces noticeable changes in materials (Goldman, 1962). These changes can be explained in several mechanisms in literature, but not all mechanisms involved are well known or understood. Most of the reported effect and mechanisms are related to cavitation phenomenon.

1.4.1.1 Cavitation phenomenon

Cavitation phenomenon is produced during the power ultrasound application in a liquid medium. Cavitation refers to the formation, growth and implosion of micro bubbles in liquids. In other words, cavitation can be defined as the formation of cavities in a liquid environment when applying negative pressure (Blitz, 1963; Gooberman, 1969; Biltz, 1971; Suslick, 1988; Raso et al, 1998a; Mason, 2012).

Although cavitation phenomenon is explained as many authors, the explanation of Suslick (1988; 1989) especially gained importance. This explanation includes that during the transmission of ultrasound waves in liquid media, consecutive compression and rarefaction cycles are produced. During the expansion cycle, high intensity ultrasound wave produce small bubbles that grow in a liquid. The micro bubbles reach successive volume and they implode violently. Because they cannot hold enough energy for a long time. This phenomenon is known as cavitation. The implosion phase is only possible if the right conditions of pressure and temperature are present. In other words, while microbubbles implode, temperature (approximately 5500 K) and pressure (approximately 50 MPa) inside the bubbles are reached very high values and the heating and cooling rates of them are above 1010 K/s.

Four factors assemble to produce ultrasonic cavitation (Frizzell, 1988; Gonzalez, 2003):

- A liquid medium
- Cavitation nuclei: to initiate the formation of bubbles
- A gas or gases dissolved in the liquid phase: to help the bubbles for growing critical size
- A pressure force: big enough to pull apart the molecules of the liquid medium in order to create the bubbles or cavities.

Normally, cavitation is a nucleated process; this means that it occurs at pre-existing weak point in the liquid (Kantas, 2007). Tensile strength in pure liquids is very high and very difficult to overcome (Suslick, 1989). To formation of cavitation, the huge amount of energy used for breaking the bond between molecules. Ultrasound energy is not enough for this purpose, but all liquids consist of some impurities and dissolved gases, which are known as cavitation nuclei. It helps to reduce tensile strength of liquids to produce successful cavitation (Frizzell, 1988; Shutilov, 1988; Leighton, 1997; Gonzalez, 2003; Condón et al., 2005; Ashokkumar et al., 2007).

Cavitation bubbles are vacuum bubbles. An inert liquid and a fast moving surface motions cause the vacuum. This vacuum causes pressure differences and cavitation begins (Hielscher, 2013). A bubble in a liquid has naturally unstable characteristic. When the bubble reaches enough large volume, it will flow and explode at a surface; while it is small, it will dissolve again into the liquid. When producing the bubbles during the cavitation, bubbles absorb the energy from single cycles (compression and rarefaction) of ultrasonic irradiation (Figure 1.15). These processes cause a dynamic balance between the vapor inside the bubble and the liquid outside. In cavitation, during the compression and rarefaction zones, ultrasound can form bubbles 100-150 microns in diameter and the shape of cavity begins with spherical and then becomes small rapidly (Suslick, 1989).

After formation of the cavity bubble, it can reach critical size when it can efficiently absorb energy from the ultrasonic. The size of the bubbles increases during each cycle. As seen in the Figure 1.15, in both high and low intensities of sonic, the cavity bubble can no longer absorb energy as efficiently, therefore without enough energy input, it can no longer sustain itself. Finally, the cavity implodes violently. These implosions provide an extreme condition for unusual chemical environment (Suslick, 1989).

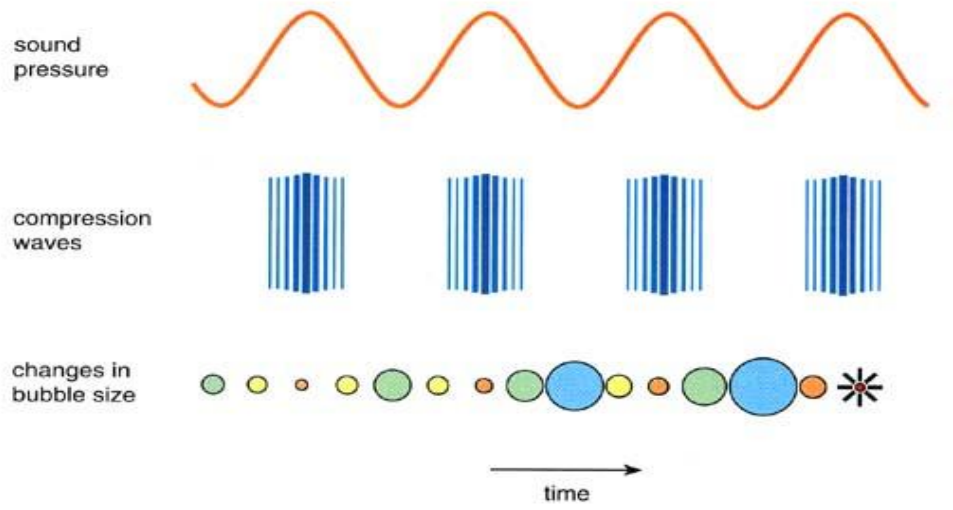

Figure 1. 15. Cavitation cycle (Suslick, 2006)

Four types of cavitation exist (Gonzalez, 2003; Milly et al., 2007). These are hydrodynamic, optic and particle and acoustic cavitation. In this thesis, acoustic cavitation and its effects determined detailly.

1.4.1.1.a Hydrodynamic Cavitation

Hydrodynamic cavitation refers to mechanical cavitation, which is the formation of gas bubbles in a fluid due to pressure fluctuations induced by mechanically. It is based on

surface tension. When the fluid flows with an accelerated speed or is forced for producing eddy currents, the fluid's kinetic energy is converted into high velocities, which cause the pressure differences. Because of this alternation of pressure levels occurs, microbubles form, grow and collapse. These mechanical procedures are called hydrodynamic cavitation (Gonzalez, 2003; Milly et al., 2007).

1.4.1.1.b Optic and Particle Cavitation

Optic and particle cavitation are related to atomic structure. When a high-intensity light beam or laser is applied to a liquid, the separation and formation of optic cavities produce. In particle cavitation, protons and neutrinos are charged particles that produce this type cavitation (Shah et al., 1999; Gonzalez, 2003).

1.4.1.1.c Acoustic Cavitation and Effects

Acoustic cavitation refers to formation and evolution of dynamic life of bubbles in liquids (Suslick, 1988, 1989; Flint and Suslick, 1991; Goldman, 1962; Kobus and Kusińska, 2008; Hielscher, 2013). The mechanical interaction between sound waves and bubbles causes the acoustic cavitation (Ashokkumar, 2007). When applied sound in a medium, it is necessary for very high intensity wave with very high frequency (ultrasonic frequency) to achieve successful cavitation. This type cavitation is known as ultrasonic (acoustic) cavitation (Shutilov, 1988). In Figure 1.16, acoustic cavitation can be seen obviously.

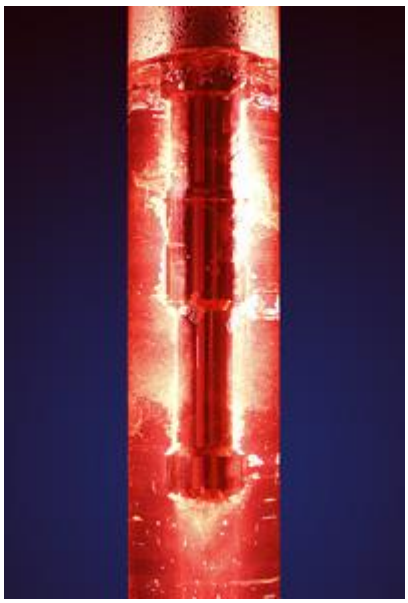

Figure 1.16. Ultrasonic cavitation (Hielscher, 2013)

According to other explanations, acoustic cavitation is the origin of some events and the main process, which is responsible for the most of sonochemical reactions in liquids. Sonochemistry is the application of ultrasound to chemical reactions and processes (Suslick et al., 1999a, 1999b; Kobus and Kusińska, 2008; Hielscher, 2013). In addition, the acoustic cavitation is responsible for not only sonochemistry but also sonoluminescence, which is emission of light (Blitz, 1963; Suslick et al., 1999a, 1999b). In addition to the heat and light produced during the cavitation, other mechanical and physical effects are also generated such as shock wave formation, turbulent motion of the liquid, acoustic noise, pressure change in the liquid (compression and rarefaction), etc (Suslick, 1989; Didenko et al., 1999; Gonzalez, 2003; Ashokkumar et al., 2007).

Two forms of acoustic cavitation are known: stable (non-inertial) and transient (inertial) according to the behaviors of bubbles (Blitz, 1963; Neppiras, 1980; Leighton, 1997; Gonzalez, 2003; Kudo and Yamamoto, 2004; Chemat et al., 2011; Condón et al., 2011).

The first form of acoustic cavitation, i.e. stable cavitation, originates from low-power ultrasound in small bubbles (Mason et al., 2003; Chemat et al., 2011; Condón et al., 2011). It is explained that the bubbles oscillate around their equilibrium position over several rarefaction/compression cycles or the bubble will be generated and its size will enter a cycle of stable growth and collapse without exploding (Gonzalez, 2003). This type cavitation takes place when the ultrasonic wave has high frequency (Mason, 1993) and low amplitude at pressures ranging from 1 to 100 kPa (Hughes and Nyborg, 1962). The stable cavitation has two type effects that are thermal and non-thermal effects on biological tissues. In thermal effect, during the vibration of micro bubble, it extracts the energy from ultrasonic irradiation. The most of the extracted power is converted into heat and temperature of the medium increases rapidly. The other effect of stable cavitation is non-thermal or mechanical effect. While ultrasonication produces the small cavity bubbles oscillate under stable cavitation, the violent collapse of the bubbles does not occur. Therefore, these small bubbles behaves as shock waves and cause strong microcurrents or microstreaming, meaning the formation of a flow around the oscillating bubble in the liquid, but the process still is not understood (Frizzell, 1988; Suslick, 1989; Scherba et al.1991; Leighton, 1997; Shah et al., 1999; Kudo and Yamamoto, 2004; Condón et al., 2011).

The second form of acoustic cavitation is transient cavitation. It is typified by the sudden expansion until critical size and then rapid collapse of the bubbles on itself. Then the cycle starts again (Suslick, 1989; Leighton, 1997; Gonzalez, 2003; Kudo and Yamamoto, 2004; Chemat et al., 2011; Condón et al., 2011). In transient cavitation, the bubbles are formed using high intensities of ultrasound waves. Transient bubbles expand through a few acoustic cycles to a radius of at least twice their initial size, before collapsing violently on compression phase (Figure 1.17)

There are three different theories of what happens after the collapse of the bubble in acoustic transient cavitation: the hot-spot (Figure 1.18), the electrical and the plasma theory. The most popular one is the hot spot theory (Suslick, 1989). During the transient cavitation, two main effects are observed such as chemical and mechanical effects (Kudo

and Yamamoto, 2004) or hot-spot theory with formation of free radicals and mechanical stress around the tissues (Suslick, 1989).

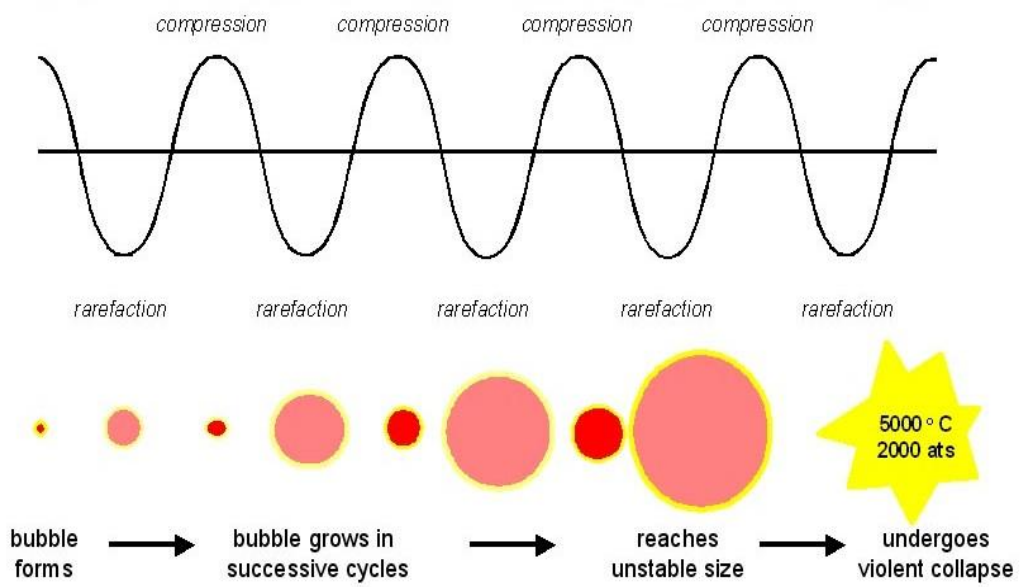

Figure 1.17. Ultrasonic bubble formation in transient cavitation (Mason, 2012)

In chemical effect, during the violently collapse of a cavitation bubble, the stored energy in the bubble gets out and the little heat can escape in the compression phase of the acoustic field. This violent collapse generates the local "hot-spot" conditions in a cold liquid. The temperature of this region is extraordinarily high. Although the medium or liquid is still cold, it will quickly press the heated cavity. The heating and cooling rates during cavitation are more than a billion degrees C per second (Suslick, 1989; Flint and Suslick, 1991; Jeffries et al., 1992; Didenko et al, 1999; Suslick et al., 1999a, 1999b; Kudo and Yamamoto, 2004). Glickstein (1960) explained this process as "cold boiling process" in the liquids. It is difficult to determine theoretically and experimentally the exact generated temperature and pressure during the cavity implosion. However, Suslick (1989) roughly reported these quantities. He claimed that a local hot spot has a temperature of predictably 5000 K, a pressure of approximately 1000 atmospheres, and a lifetime less than a microsecond. In the final step of high pressure and temperature condition, highly reactive free radicals and reagents are produced by hydrolysis inside the bubble. The medium absorbs the free radicals that cause many kinds of sonochemical effects and change in molecules (Kudo and Yamamoto, 2004).

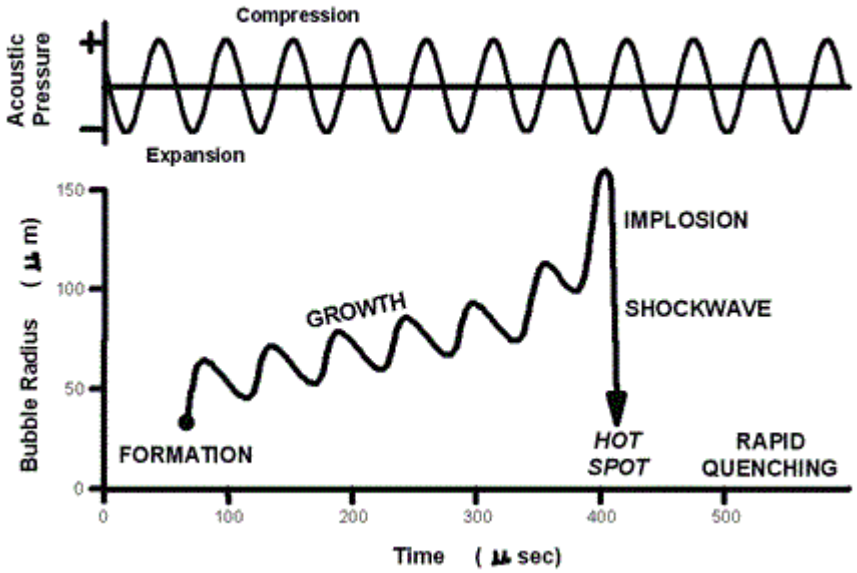

Figure 1.18. Formation of hot spot theory with bubble growth and implosion in a liquid irradiated with ultrasound (Suslick, 1989)

The mechanical effect of transient cavitation is explained that two high forces moving opposite directions caused by inertia of the medium and by pressure of the gas. In this situation, small bubble starts asymmetric contraction or is near a surface, resulting in generation of a micro jet-like small stream of medium that will hit that surface. In addition, after the implosion, the bubble rebound creates pressure and temperature. These pressure and temperature inside the bubble cause mechanical stress on the surrounding tissue and some damage (Gonzalez, 2003; Kudo and Yamamoto, 2004).

Because cavity bubbles are unbalanced bubbles, several types of bubbles do not fit in the transient and stable cavitation models. While a bubble is produced and has characteristics of other type of cavitation, the bubble is in a state known as a "threshold" bubbles. It means as the moment when the bubble changes from transient to stable cavitation or vice versa. Several factors affect the all types of cavitation (Leighton, 1997; Gonzalez, 2003; Condón et al., 2011).

1.4.1.2 Factors Affecting Ultrasonic Cavitation

The factors affecting cavitation are divided into three main categories. First of all is medium (liquid, solid or gas) characteristic properties such as; density, viscosity, diffusion constant, surface tension, vapor pressure, expansively, thermal conductivity, specific heat and amount and type of impurities present (higher concentration, larger number of bubbles). Second is thermodynamic variables which are external pressure, temperature (it starts to rice bubble formation is increased) and heat balance. Final factor is acoustic variables, frequency (lower frequency, smaller bubble and energy released),

acoustic pressure, intensity and direct and indirect ultrasound application (Alliger, 1975; Suslick, 1989; Leighton, 1997; Rahman, 1999; Gonzalez, 2003; Piyasena et al., 2003; Kantas, 2007; Ugarte-Romero et al., 2007; Kobus and Kusińska, 2008; Condón et al., 2011)

1.5 Ultrasound Equipments: Basic Units

Cavitation can be produced in different ways, such as Venturi nozzles, high-pressure nozzles, high velocity rotation, or ultrasonic transducers. In all those systems, the input energy is transformed into friction, turbulences, waves and cavitation. The fraction of the input energy that is transformed into cavitation depends on several factors describing the movement of the cavitation generating equipment in the liquid (Glickstein, 1960; Hieslcher, 2013).

Transducers generate and receive the ultrasound waves. They convert the energy of one form to that of another. The electrical energy is transformed into other kind of energies as shown in Figure 1.20.

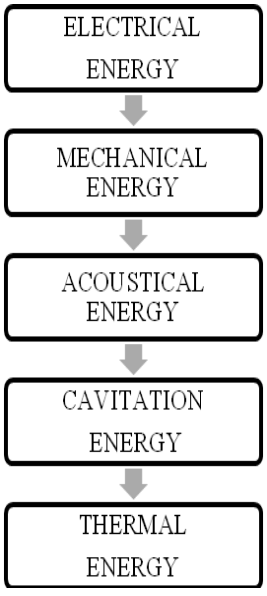

Figure 1.19. The energy transformation chain during ultrasonic treatment (Kobus and Kusińska, 2008)

In this complex energy conversion process, two main groups of equipment are required to utilize ultrasonic energy (Glickstein, 1960; Hueter and Bolt, 1960; Gooberman, 1969; Blitz, 1971):

1) A source of ultrasonic energy
    a. Mechanical transducers: Jet generators (Galton's whistle, Hartmann's whistle, Jet-edge systems and sirens)
    b. Electrical transducers or electrical source generators
2) Auxiliary equipment (control part, indicator, tank, chamber, cooling and heating parts, timer, etc.)

The acoustic energy is directly produced from mechanical transducers or jet generators. However, electrical transducers or electrical source generators producing electrical outputs in the ultrasonic frequency range convert the electrical energy to ultrasonic energy. There are two types of electromagnetic transducers in current use (Glickstein, 1960; Goldman, 1962; Blitz, 1963; Gooberman, 1969; Blitz, 1971; Gonzalez, 2003).

1) Magnetostrictive transducer: It is based on the magnetostriction of some ferromagnetic materials that causes a change in shape when magnetic field is applied (Gonzalez, 2003). It is not used for frequencies higher than about 40 kHz, but the range can be extended without any difficulty to over 100 kHz. They are reversible (Hueter and Bolt, 1960; Blitz, 1963; Blitz, 1971).

2) Piezoelectric transducer: It expands and contract when an electric field is applied. The possible frequency range extends from 20 kHz to well over 10 GHz (Blitz, 1971). In the food industry, this type transducer generally is used. It has two main effects, which are direct and inverse effects (Hueter and Bolt, 1960; Goldman, 1962; Blitz, 1963; Gooberman, 1969; Blitz, 1971; Gonzalez, 2003).

These both transducer systems include three elements (Gonzalez, 2003):

1) A generator: to convert electricity into frequency, voltage and current need by the ultrasonic system
2) A transducer: to convert the current into ultrasonic vibrations
3) A delivery system: to transfer the ultrasonic power to the medium or material

The ultrasonic power is transferred to the medium by ultrasonic delivery systems such as ultrasonic bath and ultrasonic probe, which are used in food applications. In addition to cup horns, micro plate horns and sonoreactors are used many applications (Berlan and Mason, 1991; Gonzalez, 2003; Kobus and Kusińska, 2008; Hielscher, 2013). When comparing to ultrasonic bath and ultrasonic probe, it has some advantages (Pugin, 1987; Capelo et al., 2004; Priego-Capote and Luque de Castro, 2004; Kobus and Kusińska, 2008):

1) Uniform distribution of ultrasonic energy
2) Focusing energy on localized sample zone and more efficient cavitation
3) Power control possibility and does not change over time
4) Experimental repeatability and reproducibility

1.6 Application of Ultrasound

The first commercial application of ultrasound was in the area of underwater detection around in 1900 (Glickstein, 1960; Gonzalez, 2003). During the First World War, Langevin developed a method for detecting submarines by use of underwater sound with quartz transducers. This investigation was called first studies related to ultrasonic acoustics (Glickstein, 1960; Blitz, 1971; Mason, 2012). In 1917, Lord Rayleigh described the first mathematical model for cavitational collapse predicting enormous local temperatures and pressures (Suslick, 1988; Mason, 2012). Richard and Loomis (1927) published the first paper on chemical effects of ultrasound. Harvey and Loomis (1929) first reported on the lethal effects of ultrasound on microorganisms.

Between 1930 and 1950, a number of basic discoveries in ultrasonic area were made. Ultrasound has been applied in cleaning, emulsification, processing and many similar applications and sonoluminescence effect was observed (Glickstein, 1960; Blitz, 1971; Mason, 2012). Noltingk and Neppiras (1951) mentioned hot spot process in cavitation for the first time in 1950. Since 1980, the sonochemistry effect of ultrasound was noticed (Mason, 2012).

Application of thermal treatment after ultrasound decreased the amount of several thermo resistance bacterial spores and thermoduric bacteria (Burgos et al., 1972; Ordóñez et al., 1984; Sanz et al., 1985). Ordóñez et al. (1984) firstly used thermosonication (ultrasound under moderate heat treatment). In the United Kingdom, the first international meeting devoted to sonochemistry was held in 1986 (Berlan and Mason, 1991). Earnshaw et al. (1995) discovered the inactivation effect of ultrasound on fish in the underwater. In addition, Sala et al. (1995) first indicated that microbial inactivation by high power ultrasound under pressure at nonlethal (MS) and lethal temperature (MTS). Then US, TS, MS and MTS are used for microbial and enzyme inactivation in food industry.

1.7 Ultrasound Combinations

1.7.1 Ultrasonication

Ultrasonication (US) is operated at a controlled temperature and pressure. Only ultrasonic wave amplitude affects products. The treatment time generally takes long time during the inactivation of enzymes and microorganisms. Normally, this operation is combined with other techniques such as heat and pressure (Thakur and Nelson, 1997; Villamiel and Jong, 2000a; Kuldiloke, 2002; Entezari et al., 2004; D'Amico et al., 2006; Yaldagard et al., 2008; Sagong et al., 2011).

1.7.2 Presonication

In presonication (PS) application, the product is pretreated by ultrasonication before subjected to the other treatment. Pretreatment with US can minimize the resistance of enzymes and microorganisms (Kuldiloke, 2002).

1.7.3 Postsonication

By applying postsonication (PST), the product is treated with heat and/or pressure before being subjected to US (Kuldiloke, 2002).

1.7.4 Manosonication (MS)

Manosonication is combination of ultrasound and moderate pressure 100-300 kPa at low temperature. This application obtains the inactivation of enzymes and microorganisms (Raso et al, 1998a; Mañas et al., 2000a,2000b; Kuldiloke, 2002; Álvarez et al., 2003; Arroyo et al., 2011a; Chemat et al., 2011).

1.7.5 Thermosonication (TS)

The product is subjected to ultrasound and moderate heat simultaneously at ambient pressure. The inactivation levels of thermosonication and without ultrasound at high temperature are same. As the temperature increases through the treatment, the temperature control is required (Ordóñez et al., 1984; García et al., 1989; De Gennaro et al., 1999; Villamiel and Jong, 2000a; Kuldiloke, 2002; Wu et al., 2008; Yaldagard et al., 2008; Chemat et al., 2011).

1.7.6 Manothermosonication (MTS)

In manothermosonication method, ultrasound is combined with moderate heat and moderate pressure in order to inactivation of enzymes and microorganisms. The cavitation effect which generates from ultrasound can cause inactivation enzymes and destruction of microorganisms (López et al., 1994; López and Burgos, 1995a, 1995b; Raso et al., 1998a,1998b; Pagán et al., 1999a,1999b, 1999c; Vercet et al., Commercial application of ultrasound

Ultrasound is used in a wide range of current and future applications in medicine (cancer treatment and drug delivery), electronic, food, biomedicine, biology, chemistry, nanomaterials, painting and coating, cement, concrete, beverage, environmental protection (air, land and water) and cosmetic industry (Mason, 2012; Hielscher, 2013).

Ultrasound is characterized by sound power (W), sound intensity (W/m^2) or sound energy density (Ws/m^3). Ultrasonic application in food processing can be divided into two main categories: low and high power (low and high energy) or synonymous high frequency low energy and low frequency high energy. High frequency low energy ultrasound is used to handle or check a process or product, other category of ultrasonic is low frequency high-

energy power ultrasound, which is affected directly by a process or product (Mason et al., 1996; Kuldiloke, 2002; Knorr, 2004).

High power ultrasound causes permanent physical changes of material when it is applied. These changes are generally related to cavitation phenomenon. High power ultrasound applications in liquids are cleaning, soldering, deburring, erosion testing, cell disruption, extraction from plants, emulsification, dispersion of solids, sterilization, filtration, atomization, crystal growth, crystallization of melts, degassing, mixing, medical surgery, polymerization, depolymerization, deagglomeration and deflocculation. Other applications are plastic and metal welding, impact grinding, machining, dental descaling, and metal cutting, casting of metals, and electroplating, metal forming in solids particle agglomeration, defoaming and drying in airborne (Glickstein, 1960; Goldman, 1962; Blitz, 1963; Gooberman, 1969; Blitz, 1971; Shoh, 1975; Berlan and Mason, 1991; Mason et al., 1996; Kuldiloke, 2002; Knorr, 2004; Mason, 2012).

Low power ultrasound avoids macro sonic and nonlinear acoustic areas, which are associated with the high power ultrasound. Sound speed and attenuation coefficient measuring techniques are basic steps of small-signal ultrasound (low intensity). It is non-destructive testing such as flowmetry, thermometry, density and porosity, measurement of pressure, temperature and concentration, viscosity and thickness measurement, acoustic emission, measuring elastic properties; mechanical stress etc (Glickstein, 1960; Blitz, 1963; Gooberman, 1969; Blitz, 1971; Lynnworth, 1975; Entezari et al., 2004).

1.7.7 Application of US in Food Industry

Intensity of low energy ultrasound range is lower than 1 W/cm^2 and frequency range of high frequency is between 1 to 10 MHz (Mason et al., 1996; Villamiel and Jong, 2000a, 2000b; Knorr, 2004; Patist and Bates, 2008; Mason, 2012). It is used for non-invasive detection (process control) and for characterizing physicochemical properties of food materials. In literature, several example studies represent the using low power high frequency in food processing. For instance, stimulation of activity of living cells, surface cleaning of foods, effects on enzymes, ultrasonically assisted extraction, crystallization (Gan et al., 2006), emulsification, filtration, drying and freezing processes (Sigfusson et al., 2004), tenderization of meat, ultrasonic sensor and detection of foreign particles in food products (Coupland and Saggin, 2002; Cho and Irudayaraj, 2003; Zhao et al., 2003a), determination of apperant viscosity of beverages, fluids and oils (Blitz, 1971; Saggin and Coupland, 2001b; Choi et al., 2002; Zhao et al., 2003b), measure the temperature of liquid foods (Richardson and Povey, 1990), estimate carcass composition (Fisher, 1997), measure the concentration of syrups (Saggin and Coupland, 2001a) and juices (Contreras et al., 1992) and alcohols (Winder et al., 1970), measurement of the solids content of semicrystalline fats (McClements and Povey, 1988) and fat and solid-not-fat in foods (Ghaedian et al., 1998; Chanamai and McClements, 1999; Saggin and Coupland, 2002a), estimate particle size (Coupland and McClements, 2001), cheese manufacturing processing (Cho et al., 2001; Benedito et al., 2002), evaluation of cooked vegetables (Nielsen and Martens, 1997) and of fruit ripening (Povey, 1998; Flitsanov et al., 2000).

High power low frequency ultrasonic involves intensities between 10-1000 W/cm^2 and frequencies between 20-100 kHz (McClement, 1995). The cavitational effects of high power low frequency ultrasound cause hot spot and free radical production. It has been directly used or combined with other treatment techniques in many food-processing applications. Some examples of these studies are cleaning (Kivelä, 1996), inactivation of microorganisms (Ordóñez et al., 1984, 1987; García et al., 1989; Sala et al.,1995; Ciccolini et al.,1997; Phull et al., 1997; Raso et al., 1998a.1998b; López-Malo et al., 1999; Mañas et al., 2000a, 2000b; Villamiel and Jong, 2000a, 2000b; Guerrero et al., 2001; Seymour et al., 2002; Entezari et al., 2004; Knorr, 2004; Tongson et al., 2004; Arroyo et al., 2011a, 2011b, 2012; Lee et al., 2013) and enzymes (Vercet et al., 1997, 1999, 2001; De Gennaro et al., 1999; Kuldiloke, 2002; Cruz et al., 2006; Ercan and Soysal, 2011; Cheng et al., 2013), extraction of enzymes and proteins (Moultan and Wang, 1982; Kim, 1989; Kim and Zayas, 1991a, 1991b; Sakakibara et al., 1994), homogenization (Gaffney, 1997), increasing and determination of cheese yield (Müller, 1992), tenderization and maturation of meat (Lyng et al., 1998; Gonzalez, 2003; Jayasooriya et al., 2004, 2007), sample preparation before analysis of food components (Caballo-López and Luque de Castro, 2003; Bermejo et al., 2004; Chemat et al., 2004; Priego-Capote and Luque de Castro, 2004; Yebra et al., 2005), processing of fruit juices (Kuldiloke, 2002), freezing (Sun and Li, 2003; Zheng and Sun, 2006) and development of ice crystals in frozen foods (Chow et al., 2003), emulsification and homogenization of fruit juices, mayonnaise and tomato ketchup (Behrend and Shubert, 2001; Wu et al., 2008), extraction of spices aroma compounds and organic compounds (Vinatoru et al., 1997; Vinatoru, 2001; Bruni et al., 2002; Albu et al., 2004; Chemat et al., 2004; Entezari et al., 2004; Balachandran et al., 2006; Ince, 2011); drying and filtration of foods (Kuldiloke, 2002; Kantas, 2007) ; liquefaction of honey (Basmacı, 2010); fermentation process of yogurt (Ogasawara et al., 2006).

1.8 Microbial and Enzyme Inactivation by US, TS, MS and MTS in Food Industry

1.8.1 Inactivation of Microorganisms by US:

In 1929, Harvey and Loomis firstly reported the lethal effect of US on microorganisms. A potential microbial inactivation began in around 1960; Hughes and Nyborg (1962) mentioned that the bactericidal effect of ultrasonication generally attributes to intracellular cavitation. Earnshaw et al. (1995) discovered the ultrasonication inactivation of fish in the underwater, and then ultrasonic inactivation effect was recognized. While heat treatment followed ultrasonication, Burgos et al. (1972), Ordóñez et al. (1984), and Sanz et al. (1985) represented the decreasing amount of several thermo-resistance bacterial spores and thermoduric bacteria. Ordóñez et al. (1987), García et al. (1989), and Guerrero et al. (2001) applied simultaneously heat and ultrasound (TS) treatment in their studies. In addition, Sala et al. (1995) first indicated that microbial inactivation by high power ultrasound under pressure at nonlethal (MS) and lethal temperature (MTS).

The using different parameters such as the intensity of ultrasound and the different methodologies application do not allow investigators to compare directly most of published data in literature (Condón et al., 2011). On this basis, only general conclusions can use for determination of US effects and efficiency. Although a few researchers claim

opposite results (Scherba et al., 1991), well-known consequences related to US have been reported in literature. For instance, larger cells are more sensitive to ultrasonication (Kinsloe et al., 1954; Ahmed and Russell, 1975; Condón et al., 2011) and rod-shaped bacteria are more sensitive than coccal form (Jacobs and Thornley, 1954; Alliger, 1975). Moreover, aerobic bacteria are more resistant than anaerobic bacteria species (Alliger, 1975) and gram-positive are more resistant than gram-negative bacteria (Ahmed and Russell, 1975; Pagán et al., 1999a). Additionally, vegetative cells are more sensitive than bacterial spores (Sanz et al., 1985; Raso et al., 1998b).

The microbial inactivation effect of ultrasound depends on treatment conditions, microbial characteristics and environmental factors. These three categories are divided independently in themselves (Álvarez. et al., 2003; Mañas and Pagán, 2005)

1) Treatment conditions
    a. Treatment time (D'Amico et al., 2006)
    b. Hydrostatic pressure (Raso et al., 1998a, 1998b; Pagán et al., 1999a, 1999b, 1999c; Mañas et al., 2000a; Arroyo et al., 2011a)
    c. Ultrasonic amplitude (Raso et al., 1998a,1998b; Pagán et al., 1999a, 1999b; Mañas et al., 2000a; Entezari et al., 2004; Arroyo et al., 2011a)
    d. Temperature (Ordóñez et al., 1984; García et al., 1989; Ordóñez et al., 1987; Raso et al., 1998a, 1998b ; Pagán et al., 1999a; Adekunte et al., 2010a, 2010c)
2) Microbial characteristics
    a. Microorganism (García et al., 1989; Pagán et al., 1999a; Mañas et al., 2000a; López-Malo et al., 2005; Adekunte et al., 2010a, 2010c; Sagong et al., 2011; Lee et al., 2013)
    b. Growth phase (Arroyo et al., 2011a)
    c. Growth temperature (Arroyo et al., 2011a)
    d. Sub lethal heat treatment (Pagán et al., 1999a, 1999b; Mañas et al., 2000a, 2000b; Álvarez et al., 2003, 2006; Rodríguez -Calleja et al., 2006; Arroyo et al., 2011a)
3) Environmental factors
    a. pH (Pagán et al., 1999a, 1999b; Arroyo et al.,2011a)
    b. aw (Pagán et al., 1999a, 1999b; Álvarez et al., 2003, 2006; López-Malo et al., 2005; Arroyo et al., 2011a)
    c. Composition of the treatment medium (Ordóñez et al., 1984, 1987; García et al.,1989; Mañas et al.,2000a, 2000b; Bermudez-Aguirre and Barbosa-Cánovas, 2008; Adekunte et al., 2010b)

Sonication alone reduced the counts of microorganisms, but it needs more time to inactivate or its inactivation efficency is small at low temperature and ambient pressure when compared with combination of other techniques (D'Amico et al., 2006; Sagong et al., 2011). For example, D'Amico et al. (2006) dealt with the applicability of sonication to inactivation of *L. monocytogenes* inoculated in ultrahigh-temperature milk and *E. coli* O157:H7 in apple cider at 20 kHz, 100% power level, 150 W acoustic power and 118 W/cm^2 acoustic intensity. Samples were treated with US for 3, 6, 9, 12 and 18 min at 20 ±

2°C. After 18 min continuous flow ultrasonic treatment at 20 ± 2°C, the reductions in *L. monocytogenes* population in UHT milk and in *E. coli* O157:H7 population in apple cider were measured as 3.69 log CFU/ml and 4.7 log CFU/ml, respectively. In other research, treatment with US alone (40 kHz operating frequecy, 30 W/L power, 20 °C ambient temperature) significantly reduced the numbers of *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes* on organic fresh lettuce after 20 min treatment (Sagong et al., 2011). These two studies showed that time is needed for effective microorganisms inactivation. Besides that Entezari et al. (2004) used high intesity and low intensity US alone for removing total microorganisms in date syrup at 15 and 35 °C with 10 and 25 % from total power of ultrasonic device at constant frequency (20 kHz). High intesity waves inactivate more microorganisms than low intensity waves. High intesity ultrasound destroyed 85% of total microbial count in date syrup after 90 min. However low intesity ultrasound only destroyed 49% of that at the same time. In addition to that, Adekunte et al. (2010a, 2010c) performed the sonication alone on yeast inactivtion in tomato juice, at moderate temperature (from 32 to 45°C). Extrinsic control parameters, which were 61 μm amplitude and 7.5 min at 0.5 s pulse mode, achieved 5 log reduction of yeast population in tomato juice but the inactivation level of that relatively small at lower amplitude levels and processing time. Results indicated that amplitude and treatment time were significant for the inactivation mechanism of yeast.

The effectiveness of ultrasound treatment greatly increases with the simultaneous combination of heat treatment; similar observation have been previously reported from many researchers (Ordóñez et al., 1984, 1987; García et al., 1989; Sala et al., 1995; Guerrero et al., 2001; Cabeza et al., 2004; López-Malo et al., 2005; Bermúdez-Aguirre and Barbosa-Cánovas, 2008; Adekunte et al., 2010b; Cabeza et al., 2011). For example, Ordóñez et al. (1984) combined ultrasound of 20 kHz and 160 W using cell disrupter with heating over a range of 5 to 62 °C for the inactivation of *Streptoccocus faecium* and *Streptoccocus durans* in buffer. They found that the combination of ultrasound and mild heat was significantly more effective in inactivating these bacteria than the two methods used separately. In other study of same authors (Ordóñez et al., 1987) examined a similar treatment with TS on strain of *Staphyloccoccus aureus* suspended in 0.05 M phosphat buffer (pH 6.8) and UHT milk. TS reduced *D*-values by 63 % in the buffer as compared to the *D*-values of the heat tratment alone, and by 43% when tested in UHT milk. These two studies suggests that the ultrasonication is used with combination other techniques and would allow for reduction of processing temperature, time and inactivation rate. García et al. (1989) again used TS on spore suspensions of *Bacillus subtilis* in different temperature range of 70 to 95°C in different treatment media such as distilled water, glycerol and milk. The authors mentioned that thermosonication was more effective than ultrasound alone to reduce the spore population. They also reported that treatment medium and temperature affected the spore inactivation rate, during TS application. In glycerol and milk, the reduction of spore was lower than 1 log cycle CFU/ml, but in the distilled water, that was nearly 3 log cycle CFU/ml. The effect of TS around 100 °C was dramically declined when compared the optimum experiment temperature condition of 70 °C. In another research with related to TS in milk is performed by Bermúdez-Aguirre and Barbosa-Cánovas (2008) on *Listeria innocua* strain ATCC 51742. Four different commercial milks with different butter fat content (fat free, 1% fat, 2% fat, and whole fat) inoculated *L.innocua* strain cells from early stationary growh phase were exposed to 400

W power, 24 kHz frequency and 120 μm amplitude at constant temperature (63 ±0.5°C) for 0-30 min treatment time. The inactivation rate of initial count of 10^7 CFU/ml of *L. innocua* strain ATCC 51742 increased from low level to high level related to butter fat content. Whole butter fat content milk showed a low degree of cell inactivation; a 2.5 log reduction was achieved. When fat content declined, the inactivation was faster as in the 1% and 2% butter fat content milks, where 4.5 and 3.2 log reduction were achieved. The highest inactivation (4.9 log reduction) rate was observed in fat free milk after 30 min of treatment. This research shows that the presence of fat globules in milk creates a protective effect for the cells in medium. In another research, Adekunte et al. (2010b) worked with TS on *Cronobacter sakazakii* strain ATCC 11467 in powdered infant formula(Milupa Aptamil: First) at constant frequency of 20 kHz and temperature of 50 °C. The population of strain reduced by 6.86 $\log_{10}$ units after a period of 2.5 min for treatment amplitude equal to 61 μm. López-Malo et al. (2005) mentioned that TS inactivation of molds (*Aspergillus flavus* and *Penicillium digitatum*) into sterile broth(a_w 0.99 or 0.95 and pH 5.5 or 3.0) at 20 kHz constant frequency. Continuous operation was applied at an amplitude 60, 90 or 120 μm and at 45 – 60 °C temperature. In the evaluated temperature range, application of TS at amplitude of 90 or 120 μm decrased spore resistance, being more noticeable at temperatures ≤ 57.5 °C for *A.flavus* and ≤ 50 °C for *P.digitatum*. However the researcher observed that at temperature around 60 (for *A.flavus*) or 52.5 °C (for *P.digitatum*) ,the benefits of sonication application were reduced.

The combination of ultrasound with moderate pressure is known as manosonication. In 1992, Sala et al. (1992) designed and built a resistometer in Zaragoza University to apply high power ultrasound under pressure at nonlethal temperatures. Inactivation of *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Senftenberg by ultrasonic waves under pressure was performed by Mañas et al. (2000a). The resistance of *Salmonella* Enteritidis (ATCC 13076), *Salmonella* Typhimurium (ATCC 13311) and *Salmonella* Senftenberg 775W (ATCC 43845) to ultrasonic waves (117 μm, 20 kHz) under pressure treatment (200 kPa) at sublethal temperature (40 °C) was compared with heat treatment (60 °C) in citrate-phosphate buffer and liquid whole egg. The resistance to MS of these three serotypes in both media was very similar to compare with their resistance to heat. This means that the both resistances of these three serotypes was not affected by treatment medium. Moreover, the authors investigated the amplitude and pressure effects on inactivation MS mechanism. The decimal reduction times were measured in MS treatment (200 kPa, 20 kHz, 40 °C) at different amplitudes (35, 62, 90, 117 and 150 μm) and in MS treatment (117 μm, 20 kHz, 40 °C) at different static relative pressures (0, 50, 100, 200, and 250 kPa). When changing the amplitude, no significant differences were found on the lethal effect of MS treatment in each of the three serotypes. Additionally, the inactivation rate of MS increased exponentially when amplitude was increased linearly. In the third part of study, the pressure magnitudes increase, D_{MS} values decreased for three serotypes of *Salmonella.* Furthermore, Mañas et al. (2000b) compared to power measurements and the inactivation effect of ultrasound under pressure treatment on *L. monocytogenes* ATCC 15313 in pH 7 citrate-phosphate buffer as second study. They found a relationship between the power entering the treatment medium, measured by a calorimetric method, and the decimal reduction times of *L.monocytogenes* under different experimental conditions. The lethal effect of MS treatments could be estimated

from simple calometric measurements. Another research depending on the inactivation *Salmonella* by MS was reported by Álvarez et al. (2003). In this study, the inactivation of *Salmonella enterica* serovar Enteritidis strain ATCC 13076 by ultrasonic waves under pressure at different water activities was a phenomenon of the "all-or-nothing" type, probably due to the mechanical disruption of the cell envelopes in media with high a_ws. The microorganism was exposed to 117 μm amplitude, 20 kHz frequency under 175 kPa pressure at different temperatures (35-71 °C) in media with different a_ws. The MS resistance of Salmonella increased when the aw of the treatment media decreased. In additon, the inactivation mechanisms of heat and manosonication were different because D_{heat} and D_{MS} values were 2.60 and 0.42 min at same temperature and same a_w value, respectively or D_{heat} value was nearly six times higher than D_{MS} value. The second study of Álvarez et al. (2006) was related to Salmonella Senftenberg 775 W inactivation by ultrasonic waves (20 kHz, 117 μm) under pressure (175 kPa) treatment at sublethal temperatures (35-45 °C) in media of different water activities (0.99-0.93). The D_{MS} values hardly changed with a_w. When compared to data previously obtained (Álvarez et al., 2003) for *Salmonella* Enteritidis, while the heat resistance of this serovar in media of reduced a_w was up to 10 fold lower than that of serovar Senftenberg strain 775W, MS sensitivy of both serovars was similar. Furthermore, Rodríguez -Calleja et al. (2006) compared to three inactivation mechanisms: heat (58 °C), pulse electric field and MS (117 μm, 20 kHz, 200 kPa and 37 °C). They worked with naturally isolated *Staphylococcus aureus* in pH 7 citrate- phosphate buffer and mentioned that differences in resistance to manosonication treatments were smaller than those observed for heat treatments. Arroyo et al. (2011a) studied with *Cronobacter sakazakii* in buffer and liquid foods. The workers investigated the effects of strain of bacteria (ATCC 29544, NCTC 8155, 9238, and 9529), growth temperature (10, 20, 30,and 37 °C), pH of treatment media (4.0, 5.0, 6.0, and 7.0), water activity of treatment media (0.98, 0.96, and 0.94), incubation time (8, ,10, 24, 48, 72, 96, and 120 h) and treatment in food products (apple and orange juice, chicken and vegetable soups, and rehydrated powdered milk) on MS resistance of bacteria. The MS resistance of *C.sakazakii* was not significantly changed while strain studied, growth temperature and pH of treatment media were changed. However, the water activity, incubation time and treatment in food products affected on the MS inactivation rate of *C. sakazakii*. Moreover the study included the effects of amplitude (34, 62, 90, 117 and 145 μm) and pressure (0, 50, 100, 200,and 300 kPa) on MS resistance of bacteria and power measurements.

Sala et al. (1992) built a resistometer which is also used for both manosonication and manothermosonication (ultrasound waves under moderate pressure combined mild heat). Manothermosonication (MTS) was used for inactivation of *Bacillus subtilis* ATCC 9372 spores in pH 7 citrate-phosphate buffer by Raso et al., (1998 a). The sporicidal effect of MTS treatment depended on static pressure, amplitude of ultrasonic waves and treatment temperature. In the study, in the range of 90-150 μm amplitude, an exponential relationship was observed between the amplitude and the number of survivors. An MTS (20 kHz, 300 kPa,70 °C and 12 min)treatment at 90 μm inactivated 75% of the *Bacillus subtilis* ATCC 9372 spore population, the same treatment at 150 μm inactivated 99.9 % of this population. The MTS treatments at temperatures higher than 70 °C led to more spore inactivation. Above 500 kPa, additional increments in pressure did not increase the percentage of inactivation. The same authors (Raso et al., 1998b) also worked with

Yersinia enterocolita ATCC 9610 to determine the MTS (150 μm, 20 kHz, 300 kPa, 63 °C) resistance in pH 7 citrate-phosphate buffer. They found that D_{MTS} value of *Yersinia enterocolita* ATCC 9610 was very small when comparing heat and MS treatment. For instance, an increasing of pressure from 0 to 500 kpa at constant amplitude (150 μm) decreased the *D* value of *Y. enterocolita* eight times. At constant pressure, microbial inactivation depended on the amplitude of ultrasonic waves. Furthermore, Raso et al. (1999) described some equations which is related to the calculation of theoretical *D* values of MTS experiment. In the same research group, Pagán et al. (1999a) also studied on four different (*Streptococcus faecium, Listeria monocytogenes, Salmonella enteritidis* and *Aeromonas hydrophila*) bacteria to inactivate by heat, MS and MTS (200 kPa, 117 μm, 62 °C, 20 kHz). *L. monocytogenes*, *S. enteritidis* and *A. hydrophila* showed same inactivation behavior in the previous study (Raso et al., 1998b). The lethal effect of MTS was the result of the addition of the lethal effects of heat and manosonication. In contrary, *S. faecium* showed different behavior and has a synergistic effect. Moreover, the authors mentioned two mathematical equations to explain the effect of pressure and the amplitude on the lethality of MS and MTS treatments in gram-nevative and gram-positive bacteria. Another research of Pagán et al. (1999b) depended on the resistance of heat-shocked cells of *L. monocytogenes* aganist to MTS (200 kPa,117 μm, 20 kHz, 62-68 °C). The effect of MTS on heat-shocked cells of *L. monocytogenes* was not an additive but synergistic effect of heat and ultrasound under pressure. Non-heat shocked cells of *L. monocytogenes* like Raso et al. (1998b) non-heat shocked cells of *Y. enterocolita* observed that the effect of MTS was additive. The inactivation rates increased when pH values decreased.

1.8.2.Inactivation of Enzymes by US:

Ultrasound and its combinations have also been very effective in the inactivation of different enzymes related food quality, such as LOX, PPO, lipase, PME, protease, PE, lysozyme, whey proteins (lactalbumin and lactoglobulin), casein, alpha-amylase, PG and POD (López et al., 1994; López and Burgos, 1995a, 1995b; Vercet et al., 1997, 1999, 2002a, 2002b; López et al., 1998; De Gennaro et al., 1999; Ku et al., 2000; Villamiel and Jong, 2000b; Kuldiloke, 2002; Capelo et al., 2004; Mañas et al., 2006; Wu et al., 2008; Yaldagard et al., 2008; Ganjloo et al., 2009; Kuldiloke and Esthiaghi, 2009; Terefe et al., 2009; Ercan and Soysal, 2011; Cheng et al., 2013).

Ultrasound act alone or combined can cause enzyme inactivation through three inactivation mechanisms: (i) purely thermal, due to the enormous temperatures achieved during cavitation, (ii) free radicals generated by water sonolysis, and (iii) the mechanical forces (shear forces) created by microstreaming and shock waves. The intensity of ultrasound treatment can be enormously enhanced if it is applied under moderate heat and pressures (Burgos, 1999). The inactivation of enzymes by ultrasonic wave is dependent on time of exposure, temperature, pressure, pH of medium, and amplitude of ultrasound.

Yaldagard et al. (2008) mentioned ultrasonic power has three main effects on enzymes;

1) improving the reactions conditions,
2) decreasing the enzymes activity, and
3) instead of their inactivation, increasing the activity of free enzymes in some procedures.

Application of sonication alone need more time to inactivate enzymes. For example, Thakur and Nelson, (1997) studied the inactivation of LOX in whole soy flour suspension by ultrasonication. The time exposure, pH and the amplitude of ultrasound were other factors to influence the inactivation. The enzyme was stable at pH > 5.0 and was not inactivated even after 3 h exposure time at 20 kHz ultrasound at 22 ±1˚C. On the other hand, LOX activity decreased 70-85% when pH was lowered to 5.0 and 4.0, respectively, under similar conditions. They found that, when pH was < 5.0, the activity also decreased with increase in frequency, but after 1 h exposure time, the enzyme activity was not affected with increasing frequency (>30 kHz), above pH 5.0. Moreover, another important result was found that the inactivation of enzyme by ultrasound was irreversible process and there was no reactivation even after 24 h of storage at room temperature. In another study of enzyme inactivation by ultrasound (Villamiel and Jong, 2000b) supported these observations of Thakur and Nelson, (1997). They also suggested that effect of ultrasound without heating was very low and required long exposure times for enzyme inactivation. Ultrasound applied on milk samples was carried out at 20 kHz frequency and 120 μm amplitude wave with 150 W full power sonifier. The experiments were performed at a maximum temperature of 30 °C at different resistance times (from 40.2 to 102.3 s) to study the effect of ultrasound without effect of the heat treatment. They worked on native milk enzymes [alkaline phosphatase (AP), γ-glutamyltranspeptidase (GGTP), lactoperoxidase (LPO)], whey proteins [α-lactalbumin (α-La) and β-lacto globulin (β-Lg)] in the casein fraction. After 102.3 s exposure time of ultrasonication treatment alone in skim milk, AP and LPO enzymes were not inactivated, but GGTP enzyme activity rate was only 22%. This showed that, the exposure time decreased, the inactivation rate decreased or did not affect. Under the same condition (20 kHz, 150 W, 120 μm, 30 °C, 102.3 s), whey proteins [α-lactalbumin (α-La) and β-lactoglobulin (β-Lg)] in the casein fraction were able to denature with ultrasound. In the same study (Villamiel and Jong, 2000b), ultrasound also was combined with heat treatment at the same conditions to compare US and TS results. Application temperature of TS was 75.5°C. All types of enzymes used in the study were inactivated by TS for 102.3 s at 75.5°C. As found for AP and GGTP, higher enzyme inactivation was achieved when heating was considered to ultrasonication. For LPO enzyme, the remaining activity was observed 47.2 % in skim milk. In addition, the denaturation of whey proteins was higher, when the ultrasound treatment was performed with heat. Yaldagard et al. (2008) also reported that combination of ultrasound and heat treatment was more effective than alone sonication and synergistic effect between TT and US at the same temperature. The activity of Barley's alpha-amylase decreased linearly for all ultrasonic amplitudes (0%,20%,60% and 100%) at the end of the 15 min processing time and application different temperatures (0, 30, 50 and 70 ˚C) at constant frequency (20 kHz) and power (460 W). When the temperature was increased to 50 ˚C to introduce heat-induced inactivation, the activity was reduced to 31.980 (U/g malt) at the same cavition level. In

addition, the temperature was increased from 50° C to 70 ˚C, the enzyme activity was measured 30.530 (U/g malt). They mentioned that in order to improve the efficiency of the thermosonication treatment, the use of very high temperatures might not be very useful. This means that mild heat treatment with US may be more effective than high temperature with US treatment. In study of De Gennaro et al. (1999), the combination of high power ultrasound (20, 40, and 60 kHz) and temperature (80°C) was applied on the activity of peroxidase from horseradish suspended in water at different times. The actual ultrasonic powers varied in the range from 0 to 120 W. They compared to power and inactivation of peroxidase enzyme. With increasing ultrasonic power, *D* value decreases to an asymptotic value. They reported that, in order to improve the efficiency of theTS treatment, the use of very high power ultrasound might be useless. When used for high power (120 W) and low frequency (20 kHz) combination at 80 °C, the D_{TS} value was around 10 minutes. However, low power (40 W) and high frequency (60 kHz) combination at 80 °C, the D_{TS} value was calculated 30 minutes. The experimental results clearly indicate that the deactivating efficiency of ultrasound becomes very low with increasing power of the ultrasound waves.

Wu et al. (2007) compared that tomato juice subjected to thermosonication (TS) (24 kHz), at different amplitudes of 25, 50 and 75 μm at 60, 65 and 70°C or heat only treatments. The TS treatment at 60, 65 and 70°C for 41.8, 11.7 and 4.3 min exposure, respectively reduced PME activity by 90%. The heat only treatment at 60°C, 65°C and 70°C for 90.1, 23.5 and 3.5 min, respectively inactivated PME by 90%. TS treatment with 25-75 μm amplitude had no significant impact on the inactivation efficiency between 60 and 70°C. The *D* values obtained from TS at 60 and 65°C were much smaller than that obtained from thermal inactivation. However, this inactivation effect decreased as the treatment temperature increased at 70°C, where the *D* values for heat and TS experiment were similar. This study encouraged the before observations. Terefe et al. (2009) reported the thermosonic inactivation (20 kHz, 65 μm) of polygalacturonase (PG) and PME in tomato juice at between 50 and 75 °C. An approximately, six times increase in the PME inactivation rate at 60°C was observed when compared to the thermal inactivation rate at the same temperature. This decreased to 1.5 times as the temperature increased to 75°C and the inactivating effect of combined US and TT was synergistic. Raviyan et al. (2005) mentioned another study on tomato PME inactivation by TS. Effect of cavitation intensity in the range of 0.004-0.020 mg L^{-1} min^{-1} and temperature (50, 61 and 72°C) on PME inactivation were observed. In thermosonication treatment, D_{TS} values of PME inactivation were 7.6 and 0.4 mins at 61 and 72 °C, respectively, at constant cavitation intesity (0.005 mg L^{-1} min^{-1}). Moreover, D_{TS} values at constant temperature (72 °C) decreased (from 0.7 to 0.3 min) with increasing cavitation intensity (from 0.004 to 0.008 mg L^{-1} min^{-1}). PME inactivation rate of MTS increased when temperature and cavitation intensity increased and a strong synergistic effect was observed in the TS. According to the report of Ku et al. (2000), the enzyme inactivation mechanism of continuous ultrasound treatment was more effective than that of pulsed ultrasound treatment. TS (300 W, 20 kHz, 80˚C) experiment was applied on PE for 20 min. The residual activity of peroxidase enzyme was measured 54.64 % and 76.10 % for pulse mode 1.0 and 0.5, respectively at the same amplitude level (Ku et al., 2000). Seedless guava PE inactivation due to TT (80- 95°C) and TS (20 kHz, 25-75 % power, 80-95 °C) was studied by Ganjloo et al. (2009), who stated that US wave intensity had significant effect on PE and the

kinetic of inactivation for both treatments showed a first order model. In different study, Ercan and Soysal (2011) inactivated tomato POD by heat (63- 67°C) and US (23 kHz, 3-15 μm). The authors mentioned that TT and US inctivations of POD fitted to linear regression model and ultrasonic power increased, inactivation rate of POD increased. The effect of thermal and TS (25 kHz, 50% power) treatments on the inactivation kinetics of PPO in mushroom was studied in 55-75 °C temperature range in the study of Cheng et al. (2013). The authors found that the TT and TS inactivation kinetics of PPO followed first order kinetics and the inactivating effect of combinated process was synergistic.

MTS can reduce the treatment time or treatment temperature required for enzymatic inactivation. For example, López et al. (1994) studied with manothermosonication (20 kHz frequency, 450W power, 400 kpa pressure) on polyphenoloxidase (PPO), peroxidase and lipoxygenase in potassium buffer (pH 6.5) at four different amplitudes (ranged from 0 to 145 μm) at constant temperatures (60, 74, 123˚C). They also applied different temperatures ranged from 37 to 142.6 ˚C for different amplitudes-pressures combination such as 35 μm-400 kPa (PPO), 76 μm-400 kPa (lipoxgenase) and 145 μm- 480 kPa (peroxidase). This study has the greater the amplitude, the higher the efficiency. Three enzymes studied obeyed this rule. The enzyme destruction efficiency of the combined process greatly increases with ultrasonic wave amplitude; decimal reduction times at constant temperature decreased logarithmically with increasing amplitudes. Additively, increasing pressure caused the increasing inactivation rate of enzymes under the same experimental conditions and a synergistic effect was observed between heat and ultrasonic treatment. In other investigation of López and Burgos (1995a), they tried to inactivate lipoxygenase in phosphate buffer (pH 6.5) by MTS(20 kHz frequency, 450 W power, 0-104 μm amplitude range,120-450 kPa pressure range, 67.5- 76.3 ˚C temperature range) with some chemical additives (KCl, sugar, glycerol). Moreover, they studied effect of changing pH and enzyme concentration on MTS inactivation rate. Pressure and ultrasound wave amplitude affected the *z* value of inactivation. When temperature increased, the rate of inactivation increased. In addition, D_{MTS} values were more dependent with the pH of the medium. D_{MTS} values increased with decreasing pH. In the finally work of López et al. (1998), tomato pectic enzymes (PME and PG) were much more efficiently inactivated by MTS (20 kHz, 117 μm, 200 kPa, 62.5 °C for PME and 86 ˚C for PG) than by heat treatment (62,5 and 86 °C). *D* values of PME (62.5 °C) and PG (86 °C) were 0.85 and 0.24 minutes by MTS and 45.0 and 20.6 minutes by heat, respectively. These results proved that MTS inactivation more effective than heat treatment and the effect of heat and ultrasonic waves was synergistic in MTS inactivation of these enzymes. Furthermore, each one of enzyme sensitivity of ultrasonic irradiation was changeable depending on the chemical composition of enzyme. Vercet et al. (1999) reported another research with related to orange PME inactivation in buffer (pH 3.5) and orange juice by MTS (20 kHz, 117 μm, 200 kPa, 72 °C). The *D* values of PME inactivation were 0.8 min in buffer and 1.24 min in orange juice by MTS and 20 min in buffer and 500 min in orange juice by heat treatment. The D_{MTS} and D_{heat} values of buffer were smaller than that of orange juice. The author explained that this difference depended on the orange juice components, which was responsible for the PME protection against MTS and heat inactivation. Moreover, orange PME inactivation by MTS would also be a synergistic combination effects. In other study of Vercet et al. (2001) proved that lipases and proteases inactivation in buffer (pH 6.7) by MTS (20 kHz, 117 μm, 200-300 kPa).

The temperatures of experiments were below or above 100°C. *D* values of phospholipase enzyme inactivation in buffer were 7.6 min by heat treatment and 6.7 min by MTS, at 110°C. For α-chymotrypsin enzyme inactivation, *D* values were calculated app.7 min by heat treatment and 1.67 min by MTS at 50°C. According to these results, MTS inactivation was more effective than simple heating to inactivate enzymes. Vercet et al. (2002a) also studied the effects of MTS on tomato pectic enzymes (PME and PG). Tomato juice was subjected to MTS treatments (20 kHz, 200 kPa, 117 μm and 70 °C) or to control TT, for 1 min. TT inactivated about 38% of the initial PME activity whereas PME activity was undetecable in MTS-treated tomato juice. Heat treatments left PG activity unaffected, but MTS inactivated 62 % of total PG activity. These results suggested that MTS could be a useful technology to inactivation of enzymes. Mañas et al. (2006) investigated the inactivation of white egg lysozyme by MTS (117 μm, 200 kPa) at different temperature (30, 50 and 80 °C) in phosphate buffer (pH 6.2). MTS treatments for 7 min affected minimally the lysozyme activity, at temperatures from 30 to 50 °C. Above 50 °C, the inactivating effect of MTS increased with temperature. At 80 °C, less than 10 % of the initial activity was detected after 30 s of treatment. In this study, there was no a faster inactivation of lysozyme at lower treatment temperature (30°C). However, the inactivation of enzyme was faster the higher the temperature and times needed for a 90 % of inactivation of the enzyme by MTS. The authors stated that the inactivation of lysozyme by MTS was a synergistic process.

All these investigations showed that ultrasonication without pressure and heat treatments needed more time to inactivate microorganisms and enzymes. Furthermore, thermosonication, manosonicaiton and manothermosonication applications can reduced the treatment time or treatment temperature for inactivation of microorganisms and enzymes. Many factors affected the inactivation of ultrasonic treatments such as pH, treatment medium, types of microorganisms and enzymes, ultrasonic amplitude, pressure, temperature, etc. Although many research related to ultrasonic inactivation have been studied in many laboratory media such as buffer, soups, milk, liquid egg, etc., it is still needed to other research related to the ultrasonic effects on the inactivation of microorganisms and natural enzymes in acidic fruit juices.

1.9 Aims of The Study

The first part of this study aims to discuss the inactivation of *L. monocytogenes* and *E. coli* in fresh-packaged apple and orange juices by US and US combinations (MS and MTS). The second part analyses the inactivation of natural food enzymes PPO and PME in freshly squeezed apple and orange juices by US and US combinations (MS and MTS). Additionally, in this study, the specific objectives are;

i. to determine the MS resistance of *L. monocytogenes* and *E. coli* strains and choose the relatively most resistant strains *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 to MS inactivation
ii. to determine the effects of pH, treatment medium, ultrasonic waves amplitudes and pressures on the MS inactivation of *L. monocytogenes* and *E. coli*
iii. to achieve > 4 log reduction of target foodborne pathogens by MS and MTS

iv. to determine the effects of ultrasonic waves amplitudes and pressures on the MS inactivation of PPO and PME
v. to discuss relationships between the power measurement and MS inactivation
vi. to model the survival curves of *L. monocytogenes* and *E. coli* under MS and MTS in UHT-treated apple and orange juices using the traditional linear model,
vii. to model the survival curves of *L. monocytogenes* and *E. coli* under TT in fresh-packaged apple and orange juices using Mafart equation
viii. to model the residual acitivities of PPO and PME under MS and MTS in freshly squeezed apple and orange juices using first order kinetic model,
ix. to compare the US and thermal treatment (TT) inactivations of foodborne pathogens and enzymes for effectiveness
x. to determine the synergistic or additive effect of US and TT on inactivation of enzymes and microorganisms.

CHAPTER 2

MATERIALS AND METHODS

2.1 Thermoresistometer (TR-SC) and Ultrasonication (MS and MTS) Equipment

2.1.1 Thermoresistometer (TR-SC) Equipment

Thermal treatments were performed in the modified version of a specially designed thermoresistometer (Figure 2.1) explained in the study of Condón, et al. (1989, 1993). Zaragoza University Veterinarian Faculty Food Technology laboratories used this equipment. It consisted of an insulated stainless steel (18/8) vessel (8.5 x 12 cm- app. 300 ml capacity) with a screw cap with "O" ring. The insulation was 5 cm thick polyurethane foam polymerized "in situ" and clad with stainless steel on the outside. The upper part of the main chamber has 8 ports with screw caps to hold: a three-way valve connected to a pressure source (nitrogen cylinder), an inoculum injection port, a sampling tube which is extended during use by a silicone rubber tube, two ports to hold the two branches of the electrical heating element, a thermocouple to measure and to monitor the temperature, a deflector screen to improve turbulence and a propeller for agitation, and finally an optional thermocouple for pH-meter. Attached to the removable cap is a silicone rubber septum for sample injection and automatic syringe for the injection of the inoculum, operated by a solenoid valve, a solenoid sampling valve and sample controller are parts of the sample injection.

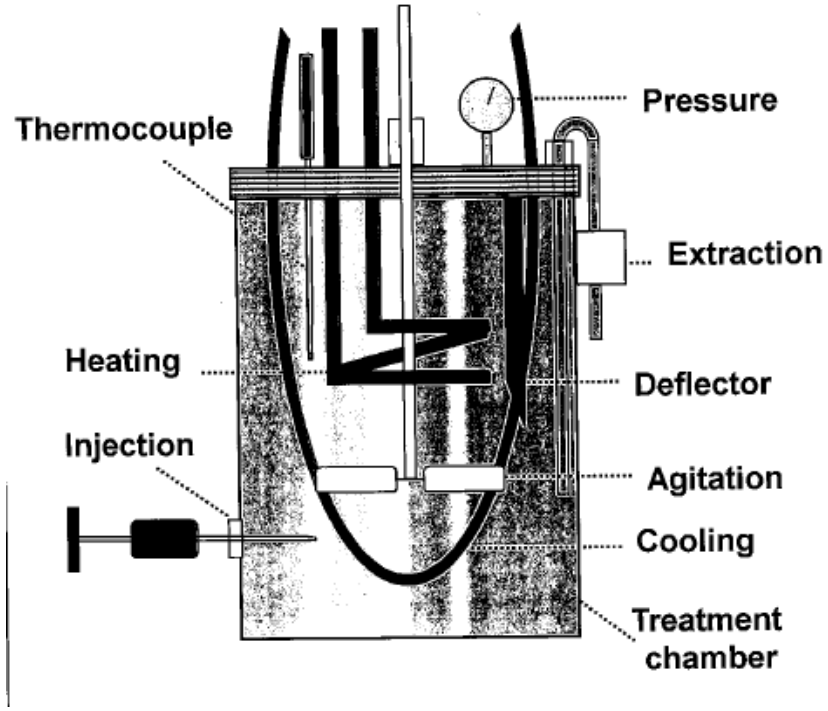

Figure 2.1. Thermoresistometer (TR-SC) equipment (Condón, et al., 1989, 1993)

Power of the heating element was controlled with a proportional controller. Power input could also be controlled manually to slow down the heating rate and thus avoid charring when viscous foods are heated. Before sampling, the contents of the sampling tube were discarded by unclamping the silicone rubber tube. During the heat treatments, temperature was constantly monitored and recorded. The instrument cooled rapidly on immersion in cold water and pressure was gauge. The medium properties are suitable for sterilization; the medium may be sterilized before inoculation by heating within the instrument and cooling to the treatment temperature.

2.1.2 MS and MTS Equipments

MTS equipment (Figures 2.2 and 2.3) in Zaragoza University Veterinarian Faculty Food Technology laboratories was specially designed a modified version of the resistometer previously described by Raso, et al. (1998a).

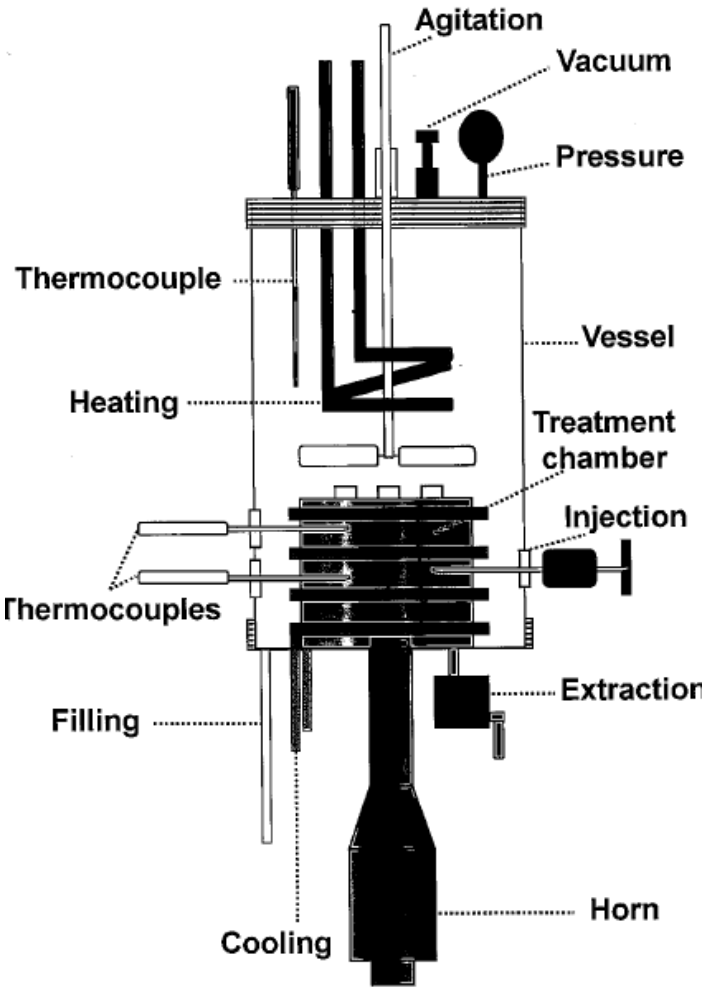

Figure 2. 2. Manothermosonication equipment (Raso, et al., 1998a)

This MTS resistometer, built of stainless steel and Teflon, consisted of a 700- ml capacity of vessel with removable top and bottom caps with "O" rings. It keeps the caps in place by stainless steel rings screwed onto the wall of the main vessel. The upper cap, as in the original instrument, had a pressure relief valve and connections to vacuum and pressure. This cap also had a pressurized housing to hold, when required, a pH electrode and held an agitation shaft, a 1,200-W heating element, and a temperature sensor.

The bottom cap had a filling and emptying tube with a valve and a cooling coil to dissipate heat generated by ultrasound. A small treatment vessel (app. 23 ml capacity) was screwed onto this cap. This small treatment vessel connected to a capillary sampling tube that had a solenoid-sampling valve activated by a timer. This vessel also had two one-way inverted sense valves in the top and three hermetical ports with "O" rings in the wall to hold a temperature sensor, a tube connected to a manometer, and an automatic injection syringe holder. One of the valves allowed the filling of the vessel by vacuum through the sampling tube before the experiment started, and the other allowed refilling it with medium from the main vessel after every sampling. Thus the medium in the

treatment chamber is diluted every taking sample. However, this dilution rate is negligible because of small amount of sampling. The bottom of the chamber was reached by the tip of the sonication horn of an ultrasound generator held in place by a housing screwed on to the bottom cap of the main vessel. In this investigation, a 450 W Digital Sonifier ultrasonic generator with a constant frequency of 20 kHz (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) was used (Figure 2.3). This constant-frequency instrument keep the amplitude constant by automatically supplying the amount of power needed to maintain the set amplitude (Raso, et al., 1998a).

Figure 2.3. MTS equipment with cooling tank and ultrasonic generator

Pressure, which is gauge, was supplied by means of nitrogen cylinder and was monitored by the manometer of the treatment vessel. Temperature control during ultrasonication experiments was achieved by circulating cold glycerol which is cooled under the 0 °C degree (-14 °C) by a cooling coil (Figure 2.3). MTS equipment can also be used for US and MS treatments.

2.2 Power Measurement Experiments

To determine the efficiency of ultrasound process, it is necessary to know acoustical energy introduced and absorbed in the liquid volume. The yields in cavitation processes also must be relevant to the acoustic power absorbed in the volume rather than to irradiated power (Margulis and Margulis, 2003).

A calorimetric method was used to determine the power input (W) into the treatment medium. This method is based on the temperature change with time of a mass of liquid absorbing the acoustic power (Berlan and Mason, 1991; Margulis and Margulis, 2003). In each experiment, the temperature rise was estimated from the slope of the straight line obtained during the first seconds of the experiment. Ultrasonic power delivered to the treatment medium was calculated with the following equation:

$$P = Cp \times m \times \frac{dT}{dt} \quad (2.1)$$

Where *P* is the power of ultrasonic generator (W), *Cp* is the heat capacity of the treatment medium (J/kg °C), *m* is the mass of the treatment medium (kg), and (*dT/dt*) is the temperature rise per second (°C/s). *Cp* value for orange and apple juices used were 4.82 kJ/kg °C (Tiwari, et al., 2009) and 3.89 kJ/kg °C (Lozano, 2006). A thermocouple connected to a data-logger (ref. OA2390-5S, ALMEMO®, Ahlborn, Holzkirchen, Germany) and insulated with heat-resistant silicone to avoid any external interference was used to measure the temperature of the treatment medium. Before measuring the temperature increase, the initial temperature was set at 30 ± 2 °C in all the experiments.

2.3 Inactivation of Microorganisms

2.3.1 Microorganisms and Growth Conditions

L. monocytogenes STCC 4031(ATCC 15313), STCC 4032(NCTC 11994), STCC 7467 (ATCC 19111), STCC 5366 (ATCC 19115), STCC 5672, and *E. coli* STCC 4201 (ATCC 11303) were obtained from the Spanish Type Culture Collection (STCC, Burjassot, Valencia, Spain). The strains of *E. coli* O157:H7 VTEC-Phage type 34 (Chapman et al., 1993) and *E. coli* W3110 (ATCC 27325) were kindly provided by Dr. B.M. Mackey from University of Reading (UK). During this investigation, the cultures were kept frozen at -80 °C in cryovials.

A stock plate of Tryptone Soy Agar (Biolife, Milan, Italy) supplemented with 0.6 % of yeast extract (Biolife) (TSAYE) was streaked with a loopful of microorganisms from each cryovial. Plates were incubated for 48 h at 30 °C for *L. monocytogenes* and 37 °C for *E. coli*. A single colony from the stock plate was transferred to a 10 ml flask of sterile Trytone Soy Broth (Biolife, Milan, Italy) supplemented with 0.6 % of yeast extract (TSBYE). The inoculated broth was hold overnight at 30 °C for *L. monocytogenes* and at 37 °C for *E. coli* in a rotary shaker (Selecta, mod. Rotabit, Barcelona, Spain) at 150 rpm. Flasks containing 50 mL of fresh TSBYE were inoculated with the overnight subculture up to a concentration of approximately 5×10^4 CFU/mL, and then incubated in the rotary shaker for 24 h at 30 °C for *L. monocytogenes* and 37 °C for *E. coli* to reach the stationary phase of growth (10^9 CFU/mL, approximately).

2.3.2 Treatment Media

McIlvaine citrate-phosphate buffer (Dawson et al., 1974) of pH 3.5 and 7.0, and commercially sterilized (UHT treated) apple juice (pH 3.4) and orange (pH 3.7) juices were used as treatment media. The apple (Alcampo S.A., Spain) and orange (García Carrion S.A., Spain) juices were purchased from local markets in Zaragoza, Spain.

2.3.3 Experimental Set-up

2.3.3.1 MS/MTS Treatment

MS and MTS treatments were carried out in specially designed resistometer (Raso, et al., 1998a) detailed in Section 2.1.2. A 450W Digital Sonifier ultrasonic generator (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) with a constant frequency of 20 kHz was used in this investigation. Four different wave amplitudes (46.5, 90, 110, and 130.5 μm) were used with gauge pressures of 0, 100 and 200 kPa. All MS treatments were carried out at a constant temperature (35 ± 0.2 °C). For MTS experiments, constant ultrasonic wave amplitude (110 μm) was used at a constant frequency of 20 kHz. Two different gauge pressures (100 and 200 kPa) and three different temperatures (50, 55, and 60 ± 0.2°C) were applied to measure the resistance of microorganisms.

Temperature control during the experiments was achieved by dissipating excess heat evolved during sonication by circulating cool glycerol through the cooling coil. The temperature of the treatment medium was continuously monitored by a thermocouple (NiCr-Ni sensor class 1, ref FTA05L0100, ALMEMO, AHLBORN, Germany), which was insulated with a heat-resistance silicone to ensure a constant target temperature value (± 0.2 °C).

After the stabilization of the temperature, pressure, and amplitude of ultrasonic waves, 0.2 mL of an appropriate dilution of the cell suspension was injected into the treatment vessel containing the treatment medium (23 mL) to obtain a final concentration of approximately 3×10^5 CFU/mL. After injection, samples of 0.1 mL were collected for definite time intervals, directly pour-plated, and incubated.

2.3.3.2 Thermal Treatment (TT)

The thermal treatment of microorganisms was performed in the specially designed thermoresistometer (TS-CR). Three different temperatures (50, 55 and 60 ± 0.2°C) at 200 kPa gauge pressure were separately applied to each treatment medium to determine the resistance of microorganisms. Firstly, 350 ml treatment vessel was filled with the treatment medium and, once the temperature and pressure were stabilized within 3-5 minutes; 0.2 mL of an appropriate dilution of the cell suspension was injected into the treatment vessel to obtain a final concentration of approximately 3×10^5 CFU/mL. After injection, samples of 0.1 mL were collected for definite time intervals, directly pour-plated, and incubated.

2.3.3.3 Incubation and Enumeration of Treated Samples

Collected samples were pour-plated in TSAYE and incubated for 24 h at 30 °C for *L. monocytogenes* and 37°C for *E. coli*. After incubation, Colony Forming Units (CFUs) were counted with an Improved Image Analyzer Automatic Colony Counter (Protos, Synoptics, Cambridge, UK), as described in Condón et al. (1987) (Figure 2.4).

Figure 2.4. Improved Image Analyzer Automatic Colony Counter (Protos, Synoptics, Cambridge, UK) (Condón et al., 1987)

2.3.3.4 Resistance Parameters and Statistical Analyses

Survival curves of TT, MS and MTS treatments were obtained by plotting the Log_{10} number of survivors *vs*. the treatment time (min). A mathematical model based on the Weibull distribution proposed by Mafart et al. (2002) was used for modeling these curves:

$$\log_{10} S(t) = (-t/\delta)^{\rho} \quad (2.2)$$

where *S (t)* is the survival fraction, *t* is the treatment time (min), δ value is the scale factor or the time for the first decimal reduction, and ρ value is the shape factor, which indicates the profile of the survival curve (ρ<1 for concave upward curves, ρ=1 for linear curves or a first order kinetics, and ρ>1 for concave downward curves). Decimal reduction time values (*D*) were used to measure the inactivation rates. For comparison purposes, microbial resistance was expressed as 1*D* or 4*D* values i.e. the time necessary to inactivate 90% (1-log_{10} cycle) or 99.99% (4-log_{10} cycles) population, respectively.

The temperature sensitivity parameter, z-value, is defined as the number of degrees (Celsius or Fahrenheit) required to change a *D*-value by one factor of ten. Mathematically

$$\mathrm{Log}\frac{D_1}{D_2} = \frac{T_2 - T_1}{Z} \quad (2.3)$$

where T_2 and T_1 were temperatures, corresponding to decimal reduction times D_2 and D_1 respectively. The *z*-value can be obtained as the negative reciprocal slope of the regression line representing Log_{10} *D vs. T* relationship. To analyze the temperature

sensitivity of *D*-values, *z*-value must be estimated using a thermal death time in thermal processing (Pilavtepe, 2007; Cabeza et al., 2010).

To fit the mathematical model to the experimental data the GraphPad PRISM® software (GraphPad Software Inc., San Diego, CA, USA) was used. Statistical analysis: ANOVA test followed by the Tukey's test (p=0.05) was used with the SPSS software (SPSS Inc., Chicago, USA) and differences were considered significant if $p \leq 0.05$. All experiments were performed in triplicate on independent days and the error bars in the figures indicate the standard deviations.

2.4 Inactivation of Enzymes

2.4.1 Raw Materials

Apples (*Malus domestica cv Delicious Gold*) of the cv. Golden Delicious and oranges (*Citrus sinensis (L.)Osbeck*) of the cv. Navelina from Valencia (Spain) were purcheased from the local market (Alcampo S.A.) in Zaragoza, Spain. Apple and orange samples were placed in freezer bags and were kept at 4°C in the refrigerator until use. The selected fruits varieties are used for industrial fruit juice production.

Fresh, non-bruised apples were washed, sorted, cleaned and squeezed with domestic food processor (Moulinex, Model AV5, Spain). The juice was immediately filtered on double-layer cheesecloth to remove foam and pulp and then obtained cloudy apple juice was exposed to the different MS, MTS and TT treatments. Similarly, fresh, non-bruised oranges were washed, sorted, cleaned, squeezed with homemade fruit juices maker (Braun Citromatic, Model 4979, Spain) and the fruit juice was homogenated with ultraturrax (Ika Labortechnik, Janke and Kunkel Gmbh, Model T 25, Stuffen, Germany) at 10000 rpm for approximately 10 seconds. The juice was immediately filtered on double-layer cheesecloth to remove foam and pulp. The obtained orange juice was under taken the different MS, MTS and TT treatments.

2.4.2 Experimental Set-up

2.4.2.1 Temperature Changes of Treatment Medium

The temperature of the treatment medium was measured with a thermocouple (NiCr–Ni sensor class 1, ref.FTA05L0100, ALMEMO®, Ahlborn, Holzkirchen, Germany) insulated with heat-resistant silicone to avoid any external interference and connected to a data-logger (ref. OA2390-5S, ALMEMO®, Ahlborn, Holzkirchen, Germany). The temperature changes in the treatment medium presented in the temperature *vs*. time (min) graphs.

2.4.2.2 MS/MTS Experiments

2.4.2.2.1. MS Experiment

The MS inactivation experiments were carried out at a constant temperature (35 ± 2˚C). Three different ultrasonic wave amplitudes (90, 110, and 130.5 μm) and three different gauge pressures (0, 100 and 200 kPa) were applied at a constant frequency (20 kHz) during the experiments. The treatment vessel was filled with Golden apple and Navelina orange juices and the large vessel was filled with McIlvaine buffer, which has the same pH with the treatment media. During MS treatment, the temperature of the medium immediately increased. Therefore, to sustain the experiment at constant temperature (35 ± 2˚C); the initial temperature of treatment medium was adjusted to 10 ± 2 °C. To avoid the temperature change between the treatment vessel and the large vessel, buffer temperature was adjusted to 25°C before filling the large vessel. During the experiment, the temperature of the sample and buffer were measured with two thermocouples. Ultrasonic generator and timer were operated at the same time to keep temperature of the medium constant. After the stabilization of the temperature, pressure, and amplitude of ultrasonic waves, sample of 0.5 mL were collected in definite time intervals into 1.5 mL eppendorf tubes submerged on ice to control enzyme activity.

2.4.2.2.2. MTS experiments

Two different gauge pressures (100 and 200 kPa) at constant amplitude (110 μm) at a constant temperature (50 and 60 ± 2˚C) were applied during the MTS experiments. The treatment vessel was filled with Golden apple and/or Navelina orange juices and the large vessel was filled with McIlvaine buffer, which was the same pH with the treatment media. During the MS treatment, the temperature of the medium immediately increased. Therefore, to sustain the experiment at constant temperature, the initial temperature of treatment medium was adjusted to 35°C for 50°C (±2 °C) and 45°C for 60°C (±2°C) in the water bath (Bunsen, Model 1620, Madrid, Spain). To avoid the temperature change between the treatment vessel and the large vessel, according to desired temperature of MTS treatment, the buffer temperature was adjusted to 40 and 50°C in the water bath, respectively, before filling the large vessel. During the experiment, the temperature of the sample and buffer were measured with two separate thermocouples. Cooling equipment, ultrasonic generator and timer were operated at the same time to keep temperature of the medium at constant. After the stabilization of the temperature, pressure, and amplitude of ultrasonic waves, sample of 0.5 mL was collected in definite time intervals into 1.5 mL eppendorf tubes submerged into ice to control enzyme activity.

2.4.2.3. Thermal Treatment

The TT experiments were carried out in a constant temperature (± 0.3 °C) agitative water bath (Bunsen, Model 1620, Madrid, Spain) at 40, 50, 60 and 70 °C for 0 - 10 min at ambient pressure. Temperature of the samples was measured by thermocouples during TT. Two test tubes containing the same amount (2 mL) of fruit juices were put in the water bath, which was set to the desired temperature. Once the samples in the test tubes reached to the desired temperature, one of the tubes was removed from the water bath and

put in the ice bath for immediately cooling. The second tube was held in the water bath through the reported process time.

2.4.2.4 Measurement of PPO Enzyme Activity

2.4.2.4.1. Buffer Solution

100 mL McIlvane buffer solution at pH 6.5 (Dawson et al., 1974) was prepared by 77.25 mL sodiumbiphosphate ($Na_2HPO_4.7H_2O$) (Merck KGaA, Darmstan, Germany) solution (a) and 22.75mL citric acid ($C_6H_8O_7$) (Merck KGaA, Darmstan, Germany) solution (b).

(a)Citric acid solution: 2.1 g $C_6H_8O_7$ (citric acid) was dissolved in 100 mL distilled water.

(b) Sodiumbiphosphate solution: 5.41 g $Na_2HPO_4.7H_2O$ (sodiumbiphosphate) was dissolved in 100 mL distilled water.

2.4.2.4.2. Substrate Solution

Catechol (Sigma-Aldrich, C 9510, St. Louise MO, USA) was used as substrate in McIlvane citrate-phosphate buffer solution (pH 6.5). Catechol substrate solution (0.5 M) was prepared by dissolving 5.5 g catechol in 100 mL McIlvane citrate-phosphate buffer solution (Ozoglu and Bayindirli, 2002).

2.4.2.4.3. Assay of PPO Activity

PPO activity was determined spectrophotometrically (Unicam UV 500 UV-Visible spectrophotometer, Cambridge, UK) at 420 nm by using catechol as the substrate. The blank cuvette contained only 1.1 mL McIlvane citrate-phosphate buffer solution with 1.9 mL 0.5 M substrate solution (catechol). To measure the activity, 0.1 mL sample was mixed with 1 mL McIlvane citrate-phosphate buffer solution with 1.9 mL substrate solution. Sample and solutions were adequately stirred for 15 s with vortex (Ika labortechnik, Staufen Germany). Once the 15 s mixing, sample was immediately poured into 3 mL transparent plastic cuvette. Absorbance values were recorded manually at every 5 sec for 3 minutes. All enzyme assays were performed at 25 ± 2 °C.

2.4.2.5. Measurement of PME Enzyme Activity

2.4.2.5.1. Substrate solution

Pectin from citrus peel (Sigma-Aldrich, P 9135, St. Louise MO, USA) as substrate and sodium chlorine was obtained from Sigma-Aldrich (St. Louise MO, USA). 0.5% (w/v) pectin was dissolved into 1 L 0.1 M NaCl, which has been heated to boiling and stirred with a magnetic stirrer. The substrate solution kept at 4°C in the refrigerator until use.

2.4.2.5.2. Buffer solution

Sodium hydroxide was purchased from Sigma-Aldrich (St. Louise MO, USA). 0.1 N and 2 N NaOH were used for adjusting pH of the solution. 0.01 M NaOH was used for buffer solution.

2.4.2.5.3. Assay of PME Activity

PME activity was determined using a modification of the method developed by Rouse and Atkins (1995). This method is easy to adjust the pH value of the samples. Many researchers have also analyzed the PME activities by this method (López et al., 1998; Vercet et al., 1999; Kuldiloke, 2002; Raviyan et al., 2005). In a 50 ml volume plastic glass, a magnetic stirrer and a 30-mL aliquot of a substrate solution containing 0.1 M NaCl and 0.5% (w/v) pectin was put and the pH of the solution was adjusted to pH 7.0 with 2N NaOH and then to pH 7.5 with 0.1 N NaOH by pH-meter (Crison, Basic 20+, Spain). According to enzyme activity, 0.01-0.05 mL untreated and treated orange juice samples were added into the solution. The pH of the pectin will decrease due to the result of PME activity. After adding the sample and decreasing the pH of the solution, the pH was quickly adjusted to pH 7.5 with 0.01 M NaOH and the time was started to record. The end of the 10 min, pH of the solution was adjusted to pH 7.5 with 0.01 M NaOH and the consuming 0.01 M NaOH was recorded. The average consumed amount of NaOH at 10 min is allowed to calculate the PME unit according to the Eq (2.4). All enzyme assays were performed at 20 ± 2°C in duplicate for each of the samples.

2.4.2.5 Inactivation parameters and Statistical Analyses

PPO and PME inactivation parameters were calculated from the experimental kinetic data. The inactivation kinetics of enzymes is usually described by a first order reaction (López et al., 1994; Terefe et al., 2009). In food processing, first order reactions are commonly described by pertinent parameters (*D* and *z* values) (López et al., 1994; Mir et al., 2006; Terefe et al., 2009).

PME units (PEU) were calculated according to the Eq. (2.4) and relative remaning PME activity was calculated using Eq. (2.5) (Kimball, 1991; Walking-Riberio et al., 2009):

$$\text{PME Unit} = \frac{(\text{ml of used NaOH})\,(\text{mol/l of NaOH})(10^3)}{(\text{ml of applied PME})(\text{time (min)})} \qquad (2.4)$$

The activity of PPO was calculated from the linear portion of the curve plotted with A_{420} *vs.*time. One unit of enzyme activity for PPO (PPOU) was defined as 0.001 A_{420} s^{-1} under the assay conditions (Oktay et al., 1995; Weemaes et al., 1997; Iyidogan, 2000) and relative remaining PPO activity was calculated using the Eq. (2.5) (Iyidogan, 2000; Walking-Riberio et al., 2009).

$$\text{Relative enzyme activity}[\%] = \frac{\text{Unit of treated juice}}{\text{Unit of untreated juice}} \text{x } 100 \qquad (2.5)$$

A *D*-value, the time required to inactivate 90 % of initial enzyme activity, is the kinetic parameter used to compare MTS and TT effects on inactivation of the selected enzymes. *D* values were calculated using Eq. 2.6 from the negative reciprocal of the slope on an inactivation curve of Log_{10} (A/A_0) *vs.* time (min) (Kuldiloke, 2002; Wu et al., 2008; Terefe et al., 2009).

$$\mathrm{Log}\left(\frac{\mathrm{A}}{\mathrm{A_0}}\right) = -\left(\frac{1}{\mathrm{D}}\right)\mathrm{t} \qquad (2.6)$$

Where *A* is the activity of enzymes at time *t*; A_0 is the initial activity of enzymes (the activity of enzymes in untreated juice); and *D* is the *D*-value.

For first order reactions, the *D* value is related to the first order reaction rate constant, k, by Eq. (2.7).

$$D = \left(\frac{2.303}{k}\right) \qquad (2.7)$$

The *z* value is defined as the temperature increase required for a 10-fold decrease of the *D* value. The *z* value is calculated using Eq. (2.8) from the negative reciprocal of the slope of the Log *D vs.* temperature curve:

$$\mathrm{Log}\, D = \mathrm{Log}\, D_{ref} - \frac{T - T_{ref}}{z} \qquad (2.8)$$

where D_{ref} is the *D* value at a reference temperature, T_{ref}.

To fit the mathematical model to the experimental data the GraphPad PRİSM software was used. Statistical analyses: ANOVA test followed by the Tukey's test (p=0.05) was used with the SPSS software and diffrences were considered significant if $p \leq 0.05$. All experiments were repeated at least twice on independent days. Analyses of all samples were run triplicate and averaged. The error bars in the figures indicate the standard deviations.

2.5 Calculation of Synergistic or Additive Effect

To determine whether an additive or a synergistic effect occurs when combining heat and ultrasound (MTS treatments), for each temperature, theoretical $4D_{MTS}$ values were calculated and compared with the experimentally obtained $4D_{MTS}$ values. Theoretical $4D_{MTS}$ values were calculated with the equation proposed by (Raso, et al., 1998a), which represent an additive effect (the lethality of the combined treatment equals the lethality of heat and ultrasound treatments acting simultaneously but independently):

$$\text{Theoretical } 4D_{\mathrm{MTS}} = \frac{(4D_{\mathrm{MS}}\, x\, 4D_{\mathrm{TT}})}{(4D_{\mathrm{MS}} + 4D_{\mathrm{TT}})} \qquad (2.9)$$

where $4D_{MS}$ and $4D_{TT}$ values were obtained from the fit of the inactivation curves for the MS and thermal treatments, respectively. Afterwards, for each treatment temperature, the magnitude of the synergistic effect was calculated with the following equation:

$$\%\ \text{Synergism} = \frac{\text{Theoretical } 4D_{MTS}\text{value} - \text{Experimental } 4D_{MTS}\text{ value}}{\text{Theoretical } 4D_{MTS}\text{value}} \text{ x } 100 \qquad (2.10)$$

CHAPTER 3

RESULTS AND DISCUSSION

The use of non-thermal treatments or the combination of these methods with other preservation technologies are gaining importance because they provide the opportunity to introduce safe and less processed products into the food market (Raso and Barbosa-Cánovas, 2003; Condón, et al., 2011). Moreover, ultrasound and the combination of ultrasound under pressure at sub-lethal (MS) and lethal temperatures (MTS) are the new non-thermal treatments proposed as an alternative to current thermal treatments to inactivate microorganisms and enzymes in food products (USFDA, 2000). Short processing times, enable to apply lower treatment temperatures, improving some functional properties of foods such as texture, flavor, color, efficiently inactivation of cells of microorganisms and enzymes and lower energy requirement are acceptable reasons for this technique in useful application of food industry (McClements, 1995; Povey, 1998; Kuldiloke, 2002; Zenker, et al., 2003; Coupland, 2004; Mañas and Pagán, 2005; Kantas, 2007; Chemat, et al., 2011).

3.1 Power Measurements

Power measurements with apple and orange juices were performed at four different amplitude values (46.5, 90, 110 and 130.5 μm) and pressures (0,100, 200 and 300 kPa) at 20 kHz frequency at ambient temperature (25 ± 2 °C). To measure the efficiency of ultrasonic power, the increasing temperature in the first seconds of the treatment was obtained from temperature *vs.* time graphs at each amplitude-pressure combinations. The ultrasonic energy is not converted completely into heat energy or cavitation. Ultrasonic generator for the input power to the transducer and for the ultrasonic power entering the treatment medium also consumes it (Mañas et al., 2000a). In other words, while some portion of ultrasound is reflected, the rest is consumed in sound to emission. When sonication medium has higher viscosity or includes a large amount of suspended solid, the conversion of energy process does not obey the estimation of power hypothesis (Berlan and Mason, 1991). In addition, because of changes in liquid properties, the cavitation activities might have altered (Baumann et al., 2005). In pure liquid or clear juice, it may be assumed that all the mechanical energy transmits to heat (Berlan and Mason, 1991). However, application of the same conditions on other liquids or juices with particles, the temperature of medium rapidly increased because of friction of boundary and interface surface of particles and of absorption of ultrasound energy and cavitation (Śliwiński, 2001; Kobus, 2006). Apple and orange juices contain suspension of small amount of pulp, pectin, some granular components, acids (Kimball, 1991; Bauman, et al., 2005). Theoretically, the presence of these particles can cause lower cavitation threshold and higher cavitation activity during the application of ultrasound than a normal treatment (Mason and Lorimer, 2002; Bauman, et al., 2005). Therefore, the temperature of treatment medium increases and power calculation would be changed related to time-temperature gradient. Moreover, orange juice has higher power values than apple juice

because the suspension of orange juice contains more particles than apple juice. The applied power calculation of apple and orange juices would also be different each other because of different specific heat capacities. Table 3.1 shows the power of ultrasound at different amplitude-pressure combinations for apple and orange juices.

Table 3.1. The relationship of power values and different amplitude – pressure combinations for apple and orange juices

Pressure (kPa)	**Amplitude (μm)**	**Power (W) for Apple juice**	**Power (W) for Orange juice**
0	46.5	24	33
	90	35	47
	110	49	49
	130.5	56	54
100	46.5	29	38
	90	46	55
	110	59	59
	130.5	78	75
200	46.5	38	50
	90	77	86
	110	96	112
	130.5	114	132
300	46.5	56	66

3.2 Microbial Inactivation

3.2.1 Variation in MS Resistances Among *L. monocytogenes* and *E. coli* Strains

The lethal effect of MS (35°C, 110 μm, 200 kPa) in McIlvaine citrate-phosphate buffer of pH 3.5 was studied in five strains of *L. monocytogenes* STCC 4031 (ATCC 15313), STCC 4032 (NCTC 11994), STCC 7467 (ATCC 19111), STCC 5366 (ATCC 19115), and STCC 5672, and three strains of *E. coli* STCC 4201 (ATCC 11303), W3110 (ATCC 27325) and O157:H7 (Table 3.2). As a reference, the lethal effect of heat treatment (60°C, 200 kPa) in citrate phosphate buffer (pH 3.5) was also studied in five strains of *L. monocytogenes* and three strains of *E. coli*. The survival curves of the eight strains under MS treatments followed a first order kinetics ($R^2 \geq 0.98$, Figures 3.1 and 3.3). Whereas the kinetics of inactivation under MS followed a first order kinetic (Scherba, et al., 1991; Pagán, et al., 1999a; 1999b; Ugarte-Romero, et al., 2007), the both species inactivation under heat treatment was not linear and Weibull model with Mafart equation was suitable for the analysis (Figures 3.2 and 3.4). Therefore, equation 2 in Materials and Methods section was used to estimate D_{MS} values. Moreover, in order to choose relatively the most MS resistant strains, D_{MS} values of strains were compared in Table 3.2. *E. coli* W 3110 and ATCC 11303 had similar linear survival curves in Figure 3.3 and regarding to the *D* values, are more sensitive to MS than *E. coli* O157:H7. Same similarity was also seen between *L. monocytogenes* strains (Figure 3.1; Table 3.2). For heat treatment, *L. monocytogenes* STCC 5672 was also relatively more resistant than other strains tested however; *E. coli* W 3110 was relatively most resistant strain among others. According to these different results, it is decided that MS resistance is the main parameter to choose the relatively most resistance strains in this study. The differences observed in Table 3.2, among the MS resistance within each species were statistically significant, indicating *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 the relatively most resistant strains ($p \leq 0.05$) to MS, with D_{MS} values of 1.74 and 0.76 min, respectively. The related calculations and statistical analysis were given in Appendix B. Although no statistically differences in MS resistance were observed for *Salmonella* spp. (Mañas, et al., 2000b) and *Cronobacter sakazakii* strains (Arroyo, et al., 2011a), Rodríguez-Calleja, et al. (2006), who studied the MS resistance of various *Staphylococcus aureus* strains, found significant differences in MS resistance. The relatively most MS resistant strains, *L. monocytogenes* STCC 5672 and *E. coli* O157:H7, were chosen to carry out the evaluation of the effect of the process parameters.

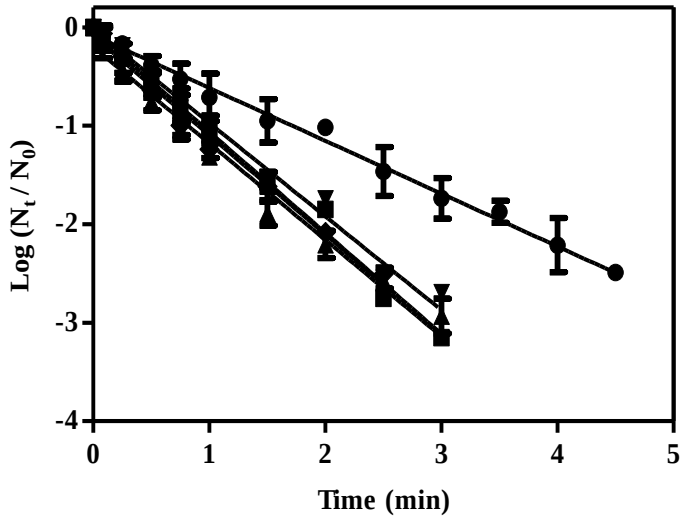

Figure 3.1. Survival curves of *L. monocytogenes* STCC 4031 (■), STCC 4032 (▲), STCC 5366 (▼), STCC 7467 (♦) and STCC 5672 (●) in McIlvaine citrate-phosphate buffer of pH 3.5 treated by ultrasound under pressure (200 kPa, 110 μm, 35°C, 20 kHz).

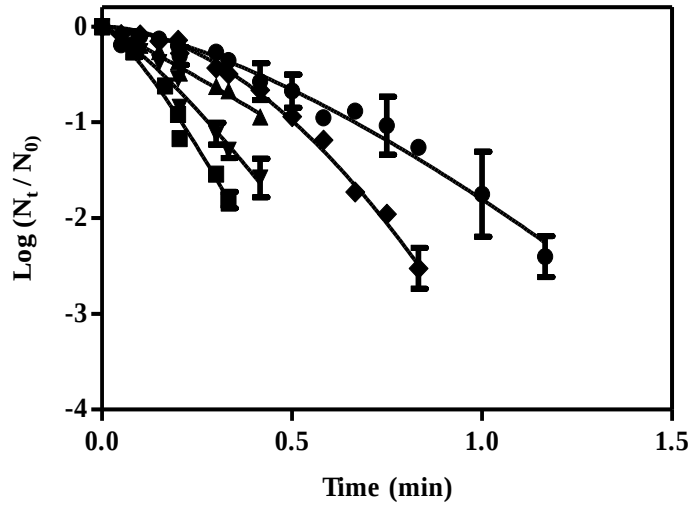

Figure 3.2. Survival curves of *L. monocytogenes* STCC 4031 (■), STCC 4032 (▲), STCC 5366 (▼), STCC 7467 (♦) and STCC 5672 (●) in McIlvaine citrate-phosphate buffer of pH 3.5 treated by TT (60°C, 200 kPa).

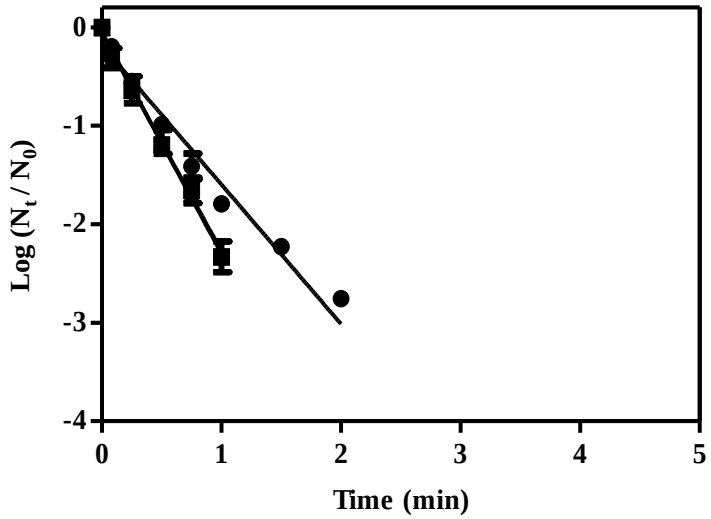

Figure 3.3. Survival curves of *E. coli* W 3110 (■), ATCC 11303(▲) and O157:H7 (●) in McIlvaine citrate-phosphate buffer of pH 3.5 treated by MS (200 kPa, 110 µm, 35 °C, 20 kHz).

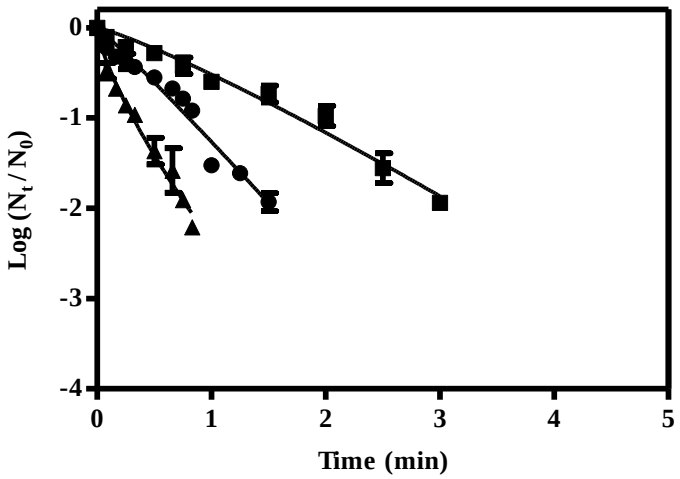

Figure 3.4. Survival curves of *E. coli* W 3110 (■), ATCC 11303 (▲) and O157:H7 (●) in McIlvaine citrate-phosphate buffer of pH 3.5 treated by TT (60°C, 200 kPa).

Table 3.2. Resistance parameters (D_{MS} and D_{TT} values) of *L. monocytogenes* and *E. coli* to MS (200 kPa, 110 µm, 35 °C) and TT (60°C, 200 kPa) in McIlvaine citrate-phosphate buffer of pH 3.5.

Microorganism	Strain	Treatment media	*D* values (min)							
			MS Treatment (35 °C, 200 kPa, 110 µm)				Thermal Treatment (60 °C, 200 kPa)			
			D_{MS}	*SD* (±)	R^2	Significant Level	D_{TT}	*SD* (±)	R^2	Significant Level
L. monocytogenes	STCC 4031	Buffer (pH 3.5)	0.96	0.02	0.99	$p \leq 0.05$	0.21	0.01	0.99	$p \leq 0.05$
	STCC 4032		1.04	0.06	0.97		0.47	0.02	0.99	
	STCC 5366		1.05	0.05	0.99		0.28	0.01	0.93	
	STCC 7467		0.99	0.02	0.98		0.50	0.01	0.99	
	STCC 5672		1.74	0.01	0.97		0.70	0.14	0.98	
E. coli	O157: H7	Buffer (pH 3.5)	0.70	0.04	0.98	$p \leq 0.05$	0.80	0.01	0.95	$p \leq 0.05$
	W 3110		0.45	0.02	0.99		1.76	0.07	0.97	
	STCC 4201		0.45	0.01	0.99		0.32	0.03	0.98	

In literature, a relationship between the effectiveness of ultrasound on inactivation and morphological features of microorganisms was reported by Jacobs & Thornley (1954); Kinsloe et al. (1954); Davies, (1959); Ahmed and Russell (1975); Alliger (1975); Raso et al. (1998a); Pagán et al. (1999a, 1999b, 1999c), and Mañas et al. (2000b). Although Scherba et al. (1991) did not find any relationship in the inactivation rate of two different kinds of microorganisms (gram-negative and gram-positive) by ultrasound, other authors (Pagán et al., 1999c; Villamiel and Jong, 2000a; D'Amico, et al., 2006) reported that gram-positive bacteria are more resistance to MS treatments than gram-negative species. Depending on the morphological features of microorganisms, which affect the microbial inactivation, it is mentioned that the differences between the cell wall compositions or structures of microorganisms affect the lethality of MS. Gram-positive organisms usually have a thicker and a more tightly adherent layer of peptidoglycans than gram-negative organisms (Scherba et al., 1991). When comparing the two pathogens used in this study, the higher resistance of *L. monocytogenes* might be relevant to bacteria morphological characteristics that are gram-positive (Davies, 1959; Ahmed and Russell, 1975), smaller size (Kinsole et al., 1954; Ahmed and Russell, 1975) and coccus shape (Jacobs and Thornley, 1954; Alliger, 1975). Regarding the interspecific differences, Gram-positive *L. monocytogenes* STCC 5672 showed higher MS resistance than Gram-negative *E. coli* O157:H7. Figures 3.5 and 3.6 show the MS resistances of *L. monocytogenes* STCC 5672 and *E. coli* O 157:H7 and allow the comparison of both microorganisms resistance. Under similar conditions, D_{MS} value of *L. monocytogenes* STCC 5672 was 2-fold higher than that of *E. coli* O157:H7. Our results also demonstrate that *L. monocytogenes* STCC 5672 (D_{MS}=1.75 min in pH 7 buffer) would display a MS resistance similar to that of other Gram-positive species such as *L. monocytogenes* (Pagán et al., 1999a, D_{MS}=1.50 min), *Streptococcus faecium* (Pagán et al., 1999b, D_{MS}=4 min), and *Staphylococcus thermophilus* (Villamiel and Jong, 2000a) and higher than that of our *E. coli* O157:H7 (D_{MS}=0.80 min in pH 7 buffer) and of other Gram negative species studied in literature such as *Salmonella* (Mañas et al., 2000b, D_{MS}= 0.84 min; Pagán et al.,1999b, D_{MS}=0.86 min), *Yersinia enterocolitica* (Raso et al., 1998a, D_{MS}=0.75 min); *Aeromonas hydrophila* (Pagán et al.,1999b, D_{MS}= 0.90 min); *Cronobacter sakazakii* (Arroyo et al., 2011a, D_{MS}=0.41 min), and *Pseudomonas fluorescens* (Villamiel and Jong, 2000a).

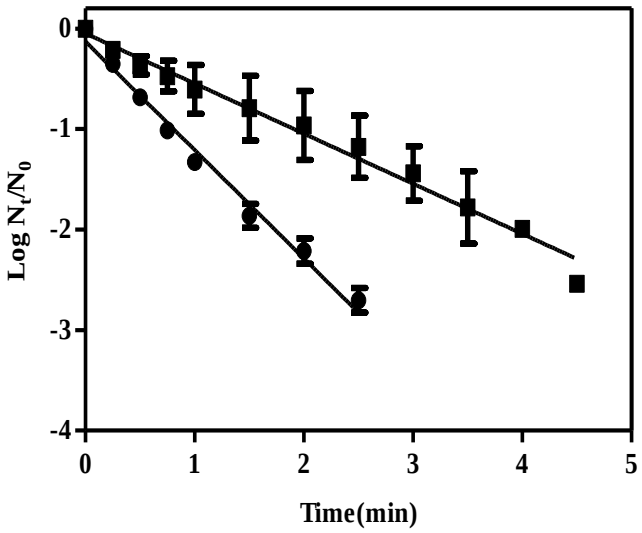

Figure 3.5. Survival curves of *L. monocytogenes* STCC 5672 (■) and *E. coli* O157:H7 (●) in apple juice (pH 3.4) treated by MS (200 kPa, 110 μm, 35 °C).

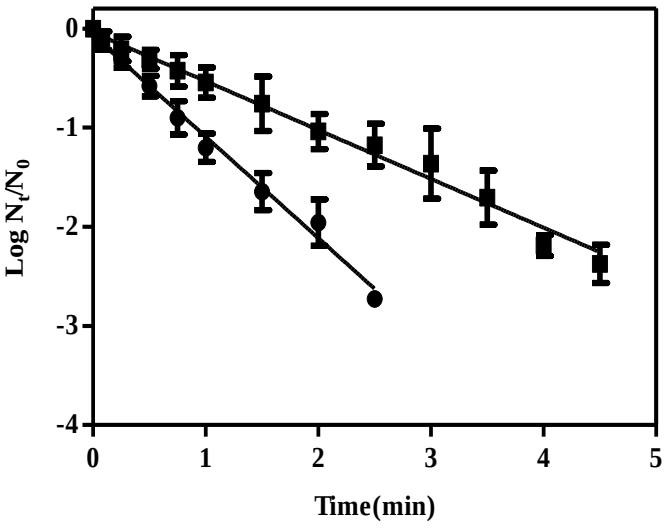

Figure 3.6. Survival curves of *L. monocytogenes* STCC 5672 (■) and *E. coli* O157:H7 (●) in orange juice (pH 3.7) treated by MS (200 kPa, 110 μm, 35 °C).

3.2.2 Effect of pH and Composition of Treatment Medium on Selected Microorganisms (Resistance in Fruit Juices)

The effect of pH on ultrasonic effectiveness is not clear. Although some authors reported higher ultrasound sensitivity at acidic pH values (Sala et al., 1995; Salleh-Mack & Roberts, 2007) and at neutral pH value (Pagán et al., 1999a), others reported no effect of pH on resistance (Guerrero et al., 2001; Arroyo et al., 2011a). Although D_{MS} values of *L. monocytogenes* in milk and McIlvaine buffer of same pH were found different each other (Pagán et al., 1999a), the MS resistance of *Cronobacter sakazakii* was not affected by variation of pH of treatment medium such as orange and apple juices, vegetable and chicken soups, buffer and rehydrated powdered milk (Arroyo et al., 2011a). Figures 3.7 and 3.8 show the inactivation by MS (35 °C, 200 kPa, 110 µm) for *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 suspended in treatment media at different pH. Citrate-phosphate buffer pH 3.5, citrate-phosphate buffer pH 7, apple juice (pH, 3.4), and orange juice (pH 3.7) were used as treatment medium. Similarly, to previous results published by Arroyo et al. (2011a), the MS resistance of *L. monocytogenes* and *E. coli* was not affected by the extreme pHs tested (3.4, 3.5, 3.7 and 7.0) ($p > 0.05$). The calculations and statistical analysis were given in Appendix B.

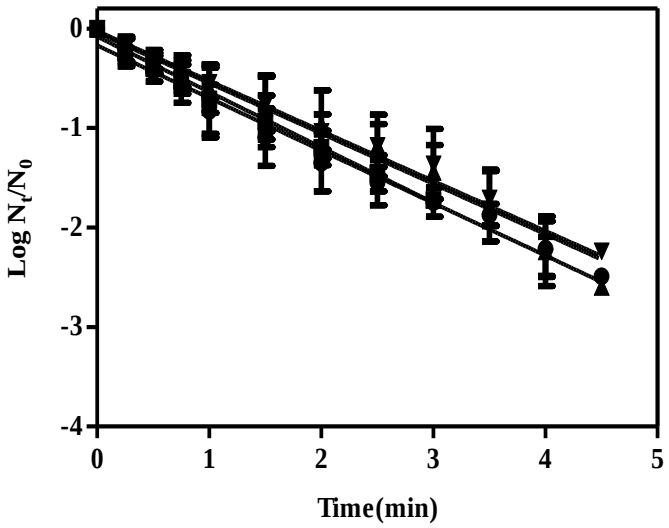

Figure 3.7. Survival curves of *L. monocytogenes* STCC 5672 in McIlvaine citrate-phosphate buffer pH 3.5(●), McIlvaine citrate-phosphate buffer pH 7(■), apple juice (pH 3.4, ▲) and orange juice (pH 3.7, ▼) treated by MS (200 kPa, 110 µm, 35 °C)

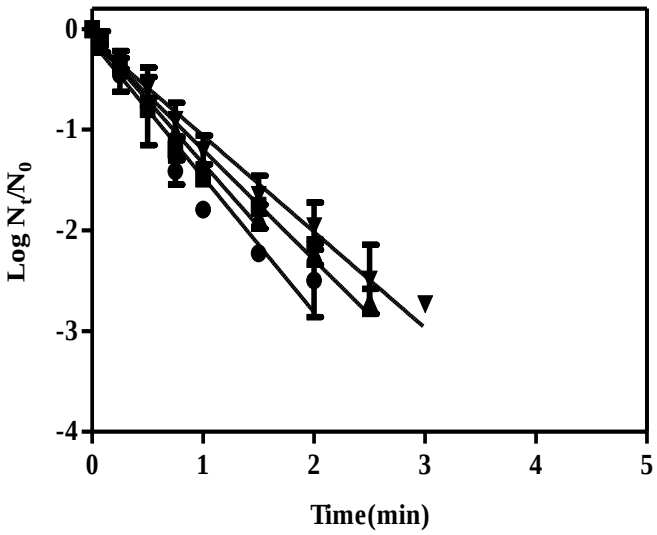

Figure 3.8. Survival curves of *E. coli* O157:H7 in McIlvaine citrate-phosphate buffer pH 3.5(●), McIlvaine citrate-phosphate buffer pH 7(■), apple juice (pH 3.4, ▲) and orange juice (pH 3.7, ▼) treated by MS (200 kPa, 110 μm, 35 °C)

Other authors have also studied the effect of the treatment medium composition on the microbial inactivation by ultrasound. Some authors found some effect of treatment media composition, for instance, Arroyo et al. (2011a) mentioned that resistance of *C. sakazakii* to MS (35 °C, 117 μm, 200 kPa) increased when liquid food products (rehydrated powdered milk, chicken and vegetable soups, apple and orange juices) were used as treatment medium in comparison to laboratory media of the same pH. Similarly, Wang et al. (2010) reported ultrasonic resistance of *Alicylobacilli* in apple juice was higher than that in buffer. Moreover, D_{MS} values of *L. monocytogenes* in milk (pH 6.7) were slightly higher (< 50%) than those obtained in McIlvaine buffer of the same pH (Pagán et al., 1999a). The different ultrasonic resistance of bacteria in food and buffer depend on their different composition. The composition of these juices may contribute to the inactivation (antibactericidal effect) or protect the microorganisms (Bauman et al., 2005). In apple juice, citric and malic acids are richer and the most known acids and Sorrells and Enigl (1990) and Han and Linton (2004) have reported that they have a bactericidal effect on microorganisms. Moreover, Wang et al. (2010) mentioned that ingredients of apple juice protected *Alicyclobacilli* from ultrasonic effect. The inactivation rate of total mesophilic aerobes in orange juice without added pulp was higher than that of in orange juice with added pulp (Valero et al., 2007). On the contrary, some other authors have found no effect of treatment media composition on bacterial resistance to ultrasound (Mañas et al., 2000b; Zenker et al., 2003). Mañas et al. (2000b) mentioned that three different serotypes of *Salmonella* had same MS resistances in liquid whole egg (pH 7.7) and citrate-phosphate buffer of pH 7.0. Besides, *E. coli* K12 DH 5α treated with ultrasound had the same *D* value in carrot juice (pH 5.9), UHT milk (pH 6.7), and pH 7.0 buffer (Zenker et al., 2003). In our study, the MS resistance of *L. monocytogenes* and *E. coli* were similar

when suspended in apple juice (pH 3.4), orange juice (pH 3.7) and McIlvaine buffer at a similar pH (3.5) ($p > 0.05$; Appendix B). As shown in Table 3.3, D_{MS} values obtained in these three media were 1.81, 1.87 and 1.74 min for *L. monocytogenes* STCC 5672 and 0.92, 0.93 and 0.76 min for *E. coli* O157:H7, respectively. Therefore, the suspension of these cells in acidic fruit juices would have a minor effect on MS resistance under the treatment conditions assayed.

Table 3.3. Resistance parameters (D_{MS}) of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 to MS (110 µm, 200 kPa, 35 °C) in four different treatment media.

Microorganisms	Treatment Media	pH	Pressure (kPa)	Amplitude (µm)	Fit Parameters			
					D_{MS} mean (min)	$SD(\pm)$	R^2	Significant Level
L. monocytogenes STCC 5672	Mcllavine buffer	3.5	200	110	1.74	0,01	0.97	NS
	Mcllavine buffer	7.0			1.75	0.04	0.98	
	Apple juice	3.4			1.81	0.08	0.98	
	Orange juice	3.7			1.87	0.08	0.99	
E. coli O157:H7	Mcllavine buffer	3.5	200	110	0.76	0.01	0.98	NS
	Mcllavine buffer	7.0			0.80	0.04	0.95	
	Apple juice	3.4			0.92	0.03	0.98	
	Orange juice	3.7			0.93	0.00	0.99	

NS; No significant

3.2.3 Microorganism Inactivation by MS and Its Relationship with Power

The energy transmitted to the medium by ultrasonic waves at different amplitudes (46.5, 90, 110 and 130.5 μm) and gauge pressures (0,100 and 200 kPa) in the two treatment media was calculated. The effect of power input on inactivation rates of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 in apple and orange juices are given in Figures 3.9 and 3.10. As mentioned before, *L. monocytogenes* STCC 5672 is more MS resistant than *E. coli* O157:H7. As can be seen that The D_{MS} values of *L. monocytogenes* have higher values than the D_{MS} values of *E. coli* at all conditions. The figures demonstrated these results. Figures 3.11 and 3.12 presented the relationship between the inactivation rates (Log D_{MS}) of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 and the energy transmitted by the ultrasound treated apple and orange juices, respectively. These graphs allow us to compare the MS inactivation rate of both microorganisms in both fruit juices. As can be seen in the figures, more power is necessary to inactivate *L. monocytogenes* STCC 5672 than to inactivate *E. coli* O157:H7 regardless of the treatment media at the same conditions.

An exponential relationship between $\log_{10} D_{MS}$ values and the energy transmitted by ultrasound waves to the treatment medium by defining a z_{MS} value has been reported (Condón et al. 2011). The treatment was carried out at different combination of amplitudes (46.5, 90, 110 and 130.5 μm) at a constant pressure (200 kPa), or of pressures (0,100, and 200 kPa) at a constant amplitude (110 μm). An exponential relationship between the D_{MS} values and the power delivered can be seen, with a z_{MS} mean value of 107 W and 121 W for *L. monocytogenes* STCC 5672 and 107 W and 116 W for *E. coli* O157:H7 in apple and orange juices, respectively (Figures 3.9 to 3.12). No significant differences were found among the slopes of the regression lines for the two species studied in both fruit juices, and consequently, between the z_{MS} values, obtaining a z_{MS} mean value of 116 W. This indicates that an increase in 116 W in the energy transferred into the apple and orange juices by ultrasound will make the inactivation rate of *L. monocytogenes* and *E. coli* increase by 10 times. Other authors have reported values in the same range for *C. sakazakii* suspended in pH 7.0 buffer (z_{MS} = 134 W) (Arroyo et al. 2011a).

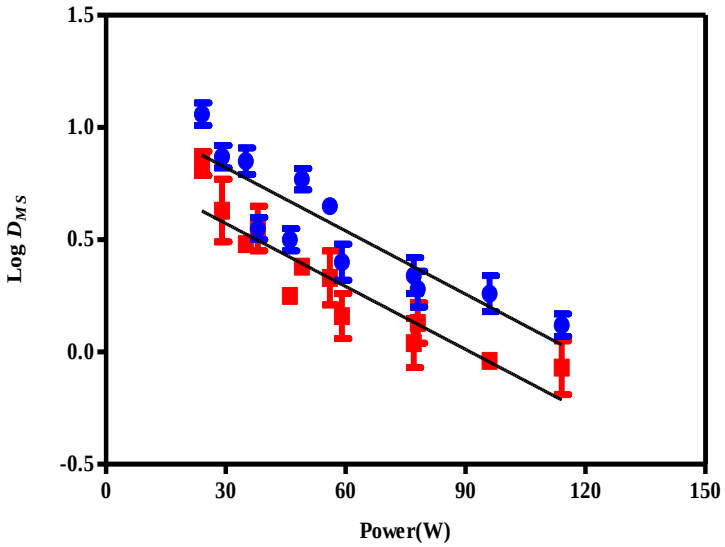

Figure. 3.9. Effect of power input (W) on the inactivation rate (Log D_{MS}) of *L. monocytogenes* STCC 5672 (●) and *E. coli* O157:H7 (■) by ultrasound treated in apple juice at different combinations of amplitudes (46.5, 90, 110 and 130.5 µm) and pressures (0,100 and 200 kPa).

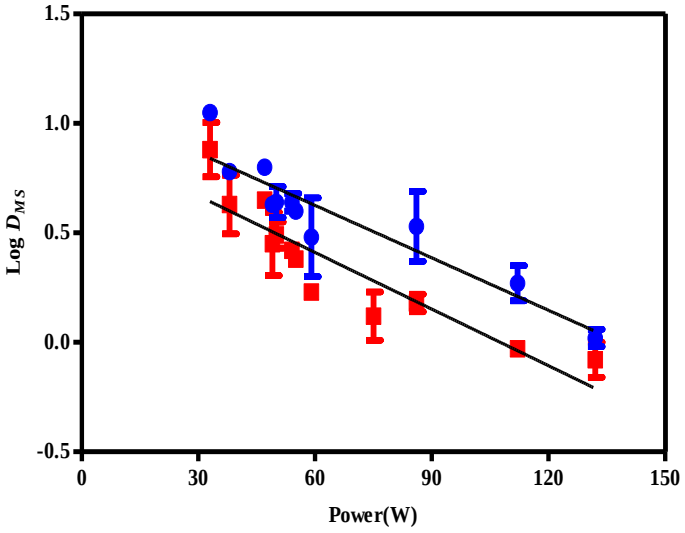

Figure. 3.10. Effect of power input (W) on the inactivation rate (Log D_{MS}) of *L. monocytogenes* STCC 5672 (●) and *E. coli* O157:H7 (■) by ultrasound treated in orange juice at different combinations of amplitudes (46.5, 90, 110 and 130.5 µm) and pressures (0,100 and 200 kPa).

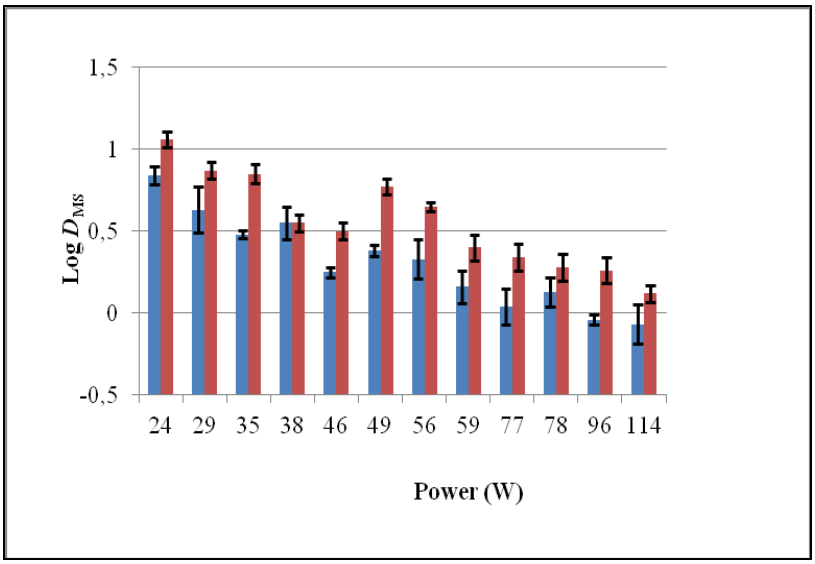

Figure 3.11. The relationship between the inactivation rates (Log D_{MS}) of *L. monocytogenes* STCC 5672 (red bar) and *E. coli* O157:H7 (blue bar) and the energy transmitted by the ultrasound treated apple juice.

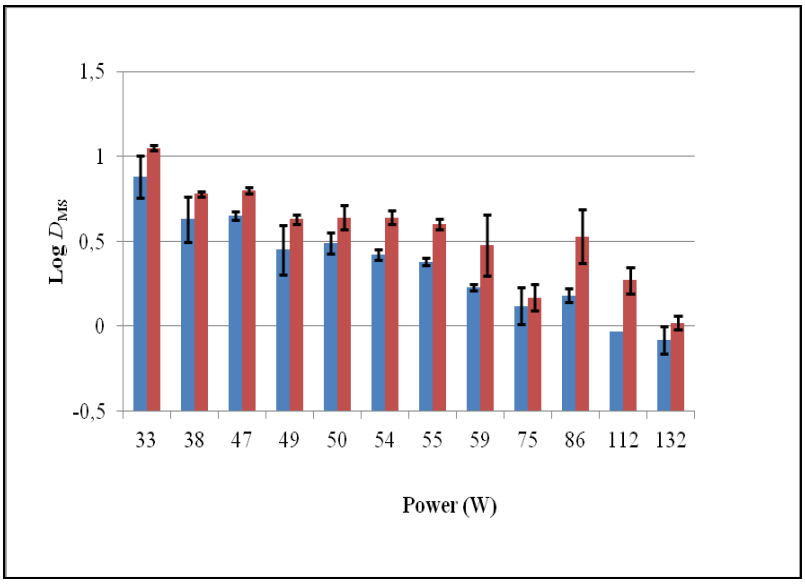

Figure 3.12. The relationship between the inactivation rates (Log D_{MS}) of *L. monocytogenes* STCC 5672 (red bar) and *E. coli* O157:H7 (blue bar) and the energy transmitted by the ultrasound treated orange juice.

3.2.4 Effect of Amplitude of Ultrasonic Waves on MS Lethal Effect

Figure 3.13 shows the effect of amplitude on the inactivation rate of *L. monocytogenes* STCC 5672 in apple juice. Log D_{MS} values of MS treatment decreased when the amplitude of ultrasonic waves increased in the range of 46.5-130.5 µm at constant pressures.

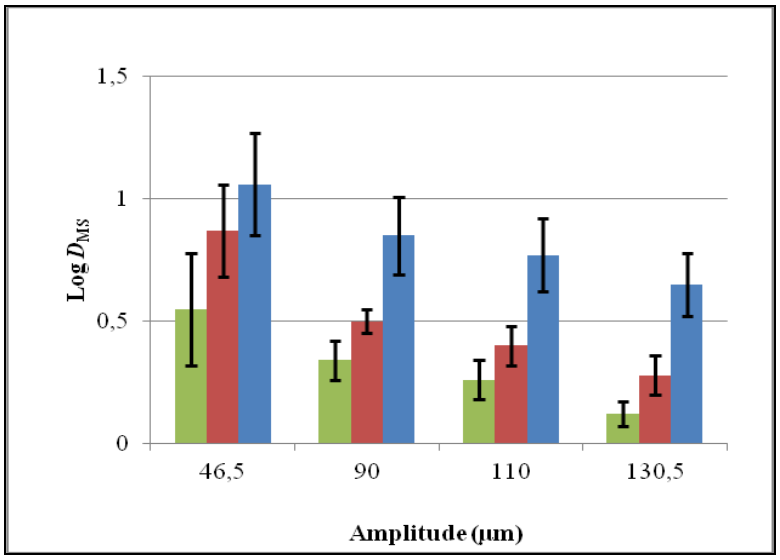

Figure 3.13. Effect of amplitude of US waves (35 °C, 20 kHz) on the inactivation rate (Log D_{MS}) of *L. monocytogenes* STCC 5672 under different pressures [0 kPa (blue bar), 100 kPa (red bar), and 200 kPa (green bar)] in apple juice.

The effect of the ultrasonic wave amplitude on the survival curves of *L. monocytogenes* STCC 5672 by MS treatments at constant pressures (0, 100, and 200 kPa) is presented in Figures 3.14, 3.15 and 3.16. The survival curves of *L. monocytogenes* STCC 5672 followed first-order inactivation kinetics with the amplitude in the range between 46.5 and 130.5 µm for the selected range of gauge pressure. Other previous reports have also described that first-order kinetic model was used to fit the linear survival curves of different microorganisms in ultrasonic treatments (Scherba et al., 1991; Pagán, et al., 1999a, 1999b; López-Malo et al., 2005; Ugarte-Romero et al., 2007; Arroyo et al., 2011a). The D_{MS} values decreased exponentially when the amplitude of ultrasonic waves increased in the range of 46.5-130.5 µm at constant pressure. Moreover, the effect of 130.5 µm amplitude was greater than the effects of other amplitudes. At 0 kPa, the MS lethality on *L. monocytogenes* increased by 61 % in apple juice when the amplitude increased from 46.5 to 130.5 µm. Similarly, the same amplitude variation increased the MS lethality on same microorganism by 74 and 62 % in apple juice at 100 and 200 kPa, respectively. Raso et al. (1998a) observed that the increase of the amplitude of ultrasonic

wave from 117 to 150 µm decreased the D_{MS} values of *Yersinia enterocolitica* by 50 % at pressures higher than 200 kPa but only to 15 % at ambient pressure. In other study, Pagán, et al. (1999a) mentioned that the D_{MS} values of *L. monocytogenes* at 0 and 300 kPa reduced approximately five-fold, when the amplitude was increased from 62 to 150 µm. Moreover, significant differences ($p \leq 0.05$) were found among the magnitude of the effects of amplitude on lethality of MS treatments at constant pressure in Appendix B. Besides, the different amplitude-pressure combinations have approximately same effect on MS inactivation rate of *L. monocytogenes* STCC 5672. As an illustration, the D_{MS} value (1.81 min) of 200 kPa pressure - 110 µm amplitude combination is nearly equal to the D_{MS} value (1.90 min) of 100 kPa pressure - 130.5 µm amplitude combination. Moreover, 46.5 µm at 100 kPa (D_{MS}=7.45 min) and 90 µm at 0 kPa (D_{MS}=7.10 min) combinations of D_{MS} values are almost the same.

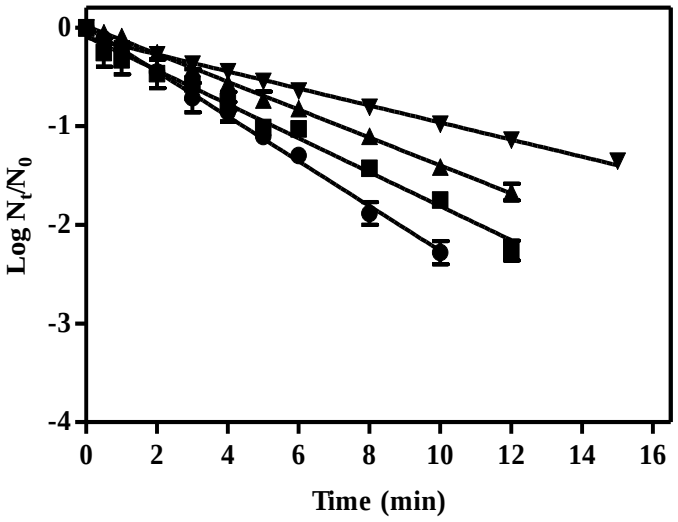

Figure 3.14. Survival curves of *L. monocytogenes* STCC5672 treated by MS (35 °C, 20 kHz) in applejuice at 0 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

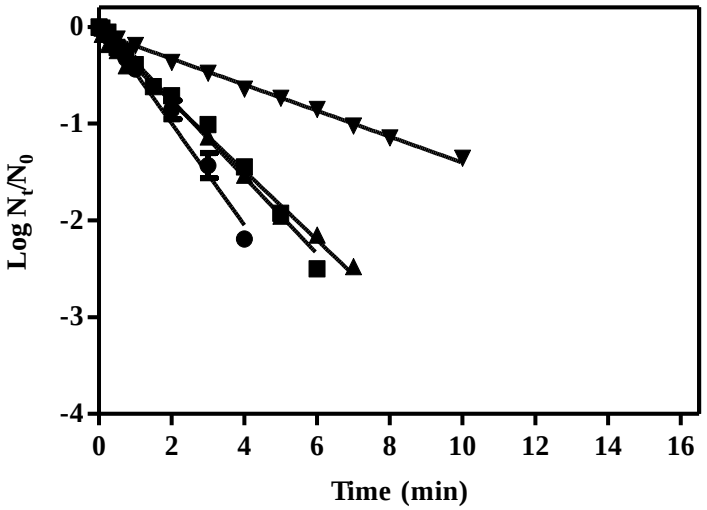

Figure 3.15. Survival curves of *L. monocytogenes* STCC5672 treated by MS (35 °C, 20 kHz) in apple juice at 100 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

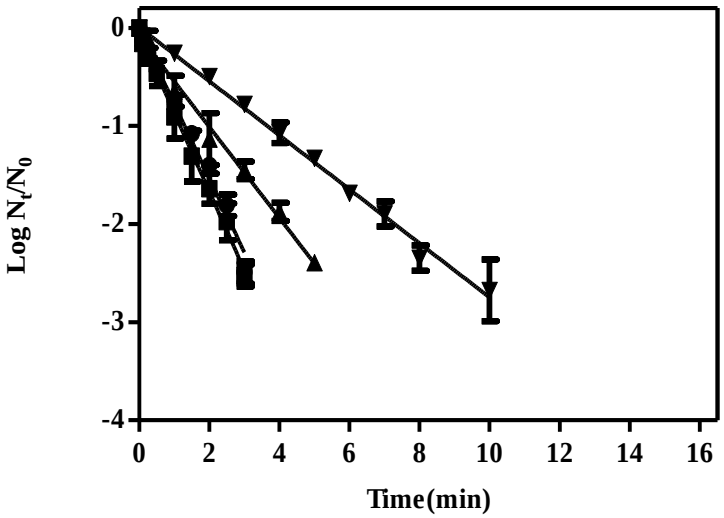

Figure 3.16. Survival curves of *L. monocytogenes* STCC5672 treated by MS (35 °C, 20 kHz) in apple juice at 200 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

Table 3.4. Resistance parameters (D_{MS}) of *L. monocytogenes* STCC 5672 treated by MS (35°C, 20 kHz) in apple juice.

Microorganism	Pressure (kPa)	Ampitude (μm)	Fit Parameters			
			D_{MS} mean (min)	$SD(\pm)$	R^2	Significant Level
L. monocytogenes STCC 5672	0	46.5	11.57	0.21	0.99	$p \leq 0.05$
		90	7.10	0.16	0.99	
		110	5.83	0.15	0.98	
		130.5	4.49	0.13	0.99	
	100	46.5	7.45	0.19	0.99	$p \leq 0.05$
		90	2.78	0.05	0.99	
		110	2.51	0.08	0.99	
		130.5	1.90	0.08	0.99	
	200	46.5	3.51	0.23	0.99	$p \leq 0.05$
		90	2.19	0.08	0.98	
		110	1.81	0.08	0.98	
		130.5	1.32	0.05	0.99	

Similar to Log D_{MS} values of *L. monocytogenes*, Log D_{MS} values of *E. coli* decreased when the amplitude of ultrasonic waves increased in the range of 46.5-130.5 μm at a constant pressure (Figure 3.17). Moreover, the $\log_{10}$ number of survivals *vs.* treatment time data obtained for four different amplitudes (46.5, 90, 110, and 130.5 μm) were plotted at constant pressures in Figures 3.18, 3.19, and 3.20. As can be seen in the figures, the survival curves of *E. coli* obtained followed first-order inactivation kinetics. Arroyo et al. (2011a) also plotted the survival curves of *Enterococcus faecium* CECT 410, *Listeria monocytogenes* CECT 4031, *Salmonella enterica serovar* Enteritidis CECT 4300, *Cronobacter sakazakii* CECT 858 and *Yersinia enterocolitica* CECT 4315 to ultrasound under pressure (35 °C, 117 μm, 200 kPa) treated in citrate–phosphate buffer of pH 7.0. First-order kinetic model was fitted to explain the linear shape of all curves in the same study. The D_{MS} values decreased exponentially when the amplitude of ultrasonic waves increased in the range of 46.5 - 130.5 μm at constant pressures. For instance, the MS lethality on *E. coli* O157:H7 increased by 70, 68 and 76 % in apple juice at 0, 100 and 200 kPa, respectively, when the amplitude increased from 46.5 to 130.5 μm. López-Malo et al. (2005) evaluated the combined effect of simultaneous application of heat treatments and low frequency ultrasound (20 kHz) at different amplitudes (0, 60, 90, and 120 μm) at ambient pressure, on *Aspergillus flavus* and *Penicillium digitatum* spore viability suspended in laboratory broth formulated at pH 3.0. The authors mentioned that when the amplitude of ultrasonic waves increased from 0 to 120 μm, the TS lethality on *Aspergillus flavus* and *Penicillium digitatum* spore increased by 81 % and 60 % in laboratory media (pH 3.0), respectively. When D_{MS} values of *E. coli* were compared to each other for each pressure, it was realized that the higher amplitude, the lower D_{MS} value was like as *L. monocytogenes*. Besides, there was significant difference between the effect of amplitudes on inactivation rate of *E. coli* O157:H7 ($p < 0.05$). The calculations and Anova analysis were given in the Appendix B.

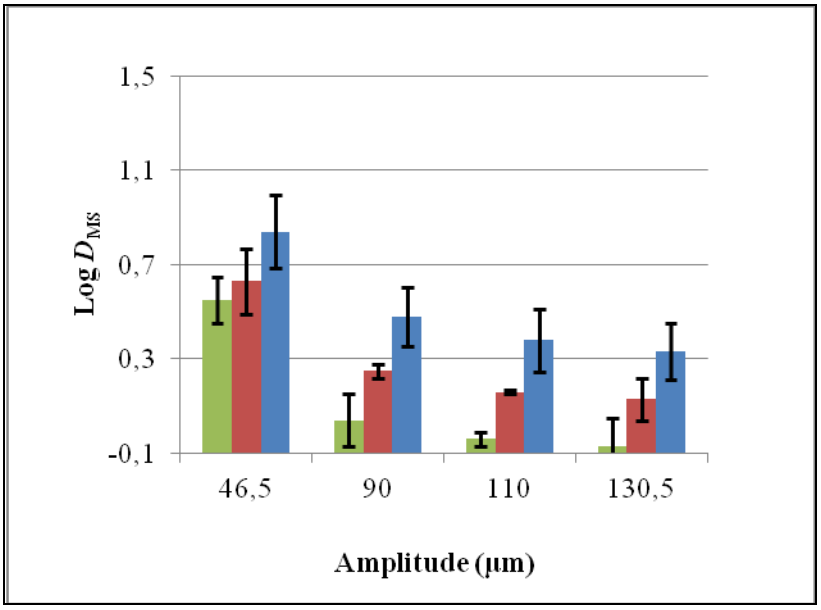

Figure 3.17. Effect of amplitude of US waves (35° C, 20 kHz) on the inactivation rate (Log D_{MS}) of *E. coli* O157:H7 under different pressures [0 kPa (blue bar), 100 kPa (red bar), and 200 kPa (green bar)] in apple juice.

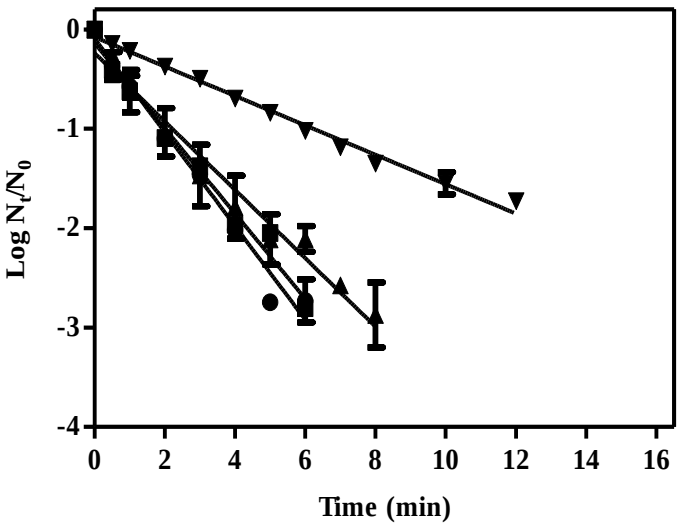

Figure 3.18. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice at 0 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

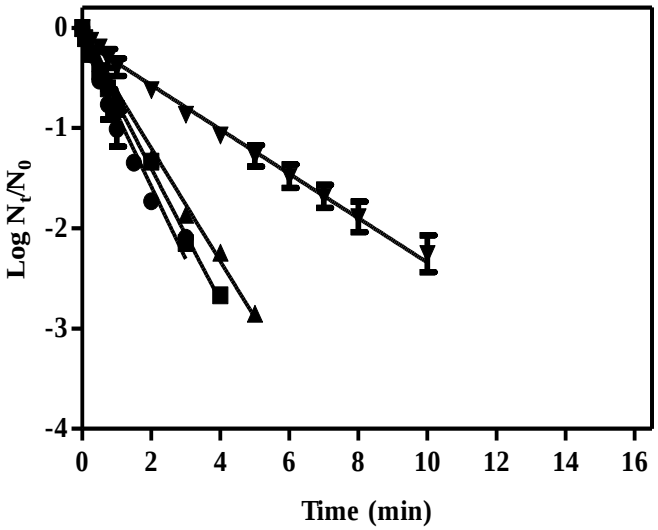

Figure 3.19. Survival curves of *E. coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice at 100 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

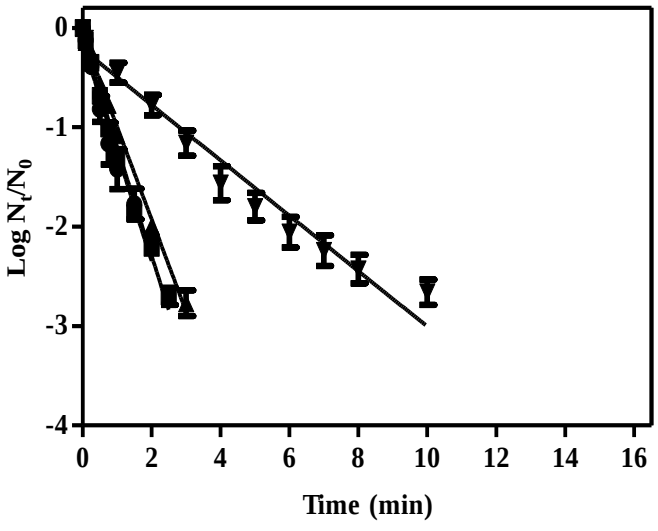

Figure 3.20. Survival curves of *E. coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice at 200 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

Table 3.5. Resistance parameters (D_{MS}) of *E. coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice.

Microorganism	Pressure (kPa)	Ampitude (μm)	Fit Parameters			
			D_{MS} mean (min)	*SD* (±)	R^2	Significant Level
E. coli O157:H7	0	46.5	6.99	0.54	0.99	$p \leq 0.05$
		90	3.05	0.26	0.98	
		110	2.41	0.33	0.99	
		130.5	2.13	0.12	0.99	
	100	46.5	4.25	0.14	0.99	$p \leq 0.05$
		90	1.77	0.03	0.99	
		110	21.46	0.10	0.99	
		130.5	1.35	0.09	0.98	
	200	46.5	3.59	0.10	0.96	$p \leq 0.05$
		90	1.09	0.11	0.99	
		110	0.92	0.03	0.98	
		130.5	0.85	0.12	0.98	

The effect of amplitude on the inactivation rate of *L. monocytogenes* STCC 5672 in orange juice are seen in Figure 3.21. Log D_{MS} values of MS treatment decreased when the amplitude of ultrasonic waves increased in the range of 46.5-130.5 µm at constant pressures.

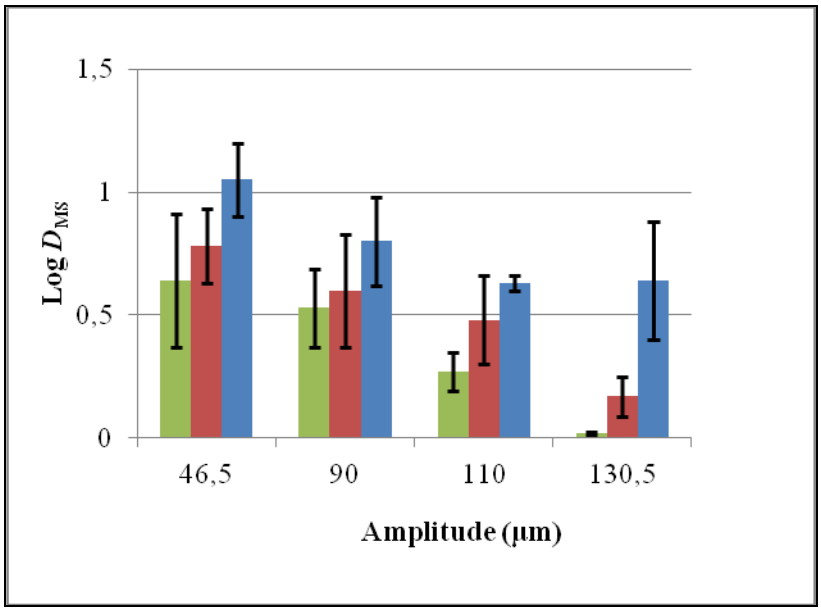

Figure 3.21. Effect of amplitude of US waves (35° C, 20 kHz) on the inactivation rate (Log D_{MS}) of *L. monocytogenes* STCC 5672 under different pressures [0 kPa (blue bar), 100 kPa (red bar), and 200 kPa (green bar)] in orange juice.

Increasing the amplitude of ultrasonic waves from 46.5 to 130.5 µm under the constant pressure in orange juice experiments caused a decrease in D_{MS} values of *L. monocytogenes* STCC 5672 similar to apple juice experiments. In all figures, the effect of amplitude was clearly seen. When ultrasonic amplitude increased from 46.5 to 130.5 µm, the D_{MS} values of *L. monocytogenes* STCC 5672 decreased. For instance, when amplitude of ultrasonic waves increased, the MS lethality of *L. monocytogenes* increased from 61, 76 and 76 % in orange juice at 0, 100 and 200 kPa, respectively. Pagán et al. (1999b) studied inactivation of *Streptococcus faecium*, *L. monocytogenes*, *Salmonella enteritidis*, and *Aeromonas hydrophila* using MS treatment. They found that the MS (200 kPa, 40 °C) decimal reduction times of the four species investigated decreased six-fold when the amplitude was increased from 62 to 150 µm in McIlvaine citrate-phosphate buffer at pH 7. The statistical analysis showed that there were significant differences between the magnitudes of ultrasonic amplitude on the lethality of MS ($p \leq 0.05$; App B).

Different amplitude-pressure combinations have shown approximately same effects on MS inactivation rate of *L. monocytogenes* STCC 5672. For instance, the D_{MS} value (1.87 min) of 200 kPa pressure - 110 µm amplitude combination is nearly equal to the D_{MS} value (1.49 min) of 100 kPa pressure - 130.5 µm amplitude combination. Moreover, 110 µm amplitude at 200 kPa pressure and 90 µm amplitude at 100 kPa pressure have virtually same D_{MS} values, 3.40 and 3.05 min, respectively. Also almost same D_{MS} values are seen in the 200 kPa-46.5 µm (D_{MS}=4.41 min) and 100 kPa-90 µm (D_{MS}= 4.01 min) combinations.

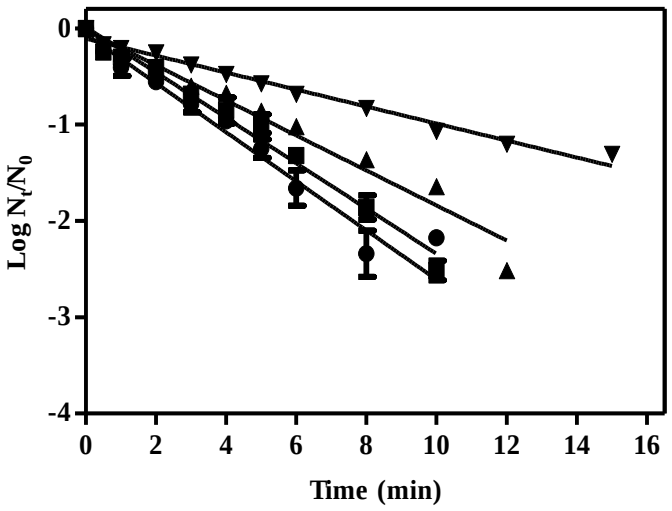

Figure 3.22. Survival curves of *L. monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice at 0 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

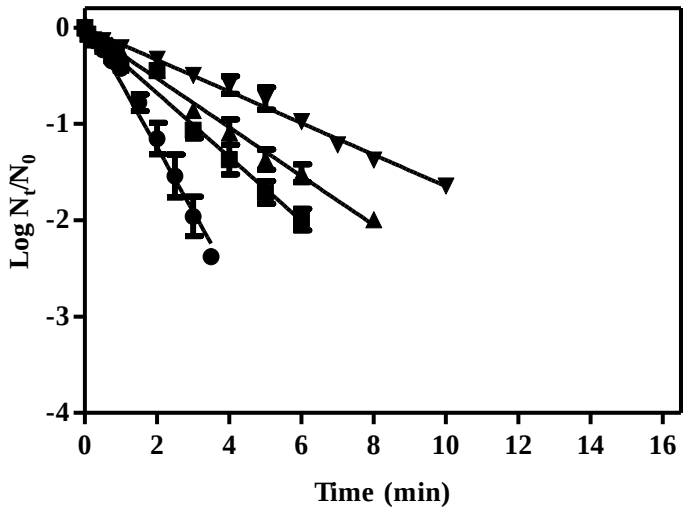

Figure 3.23. Survival curves of *L. monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice at 100 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

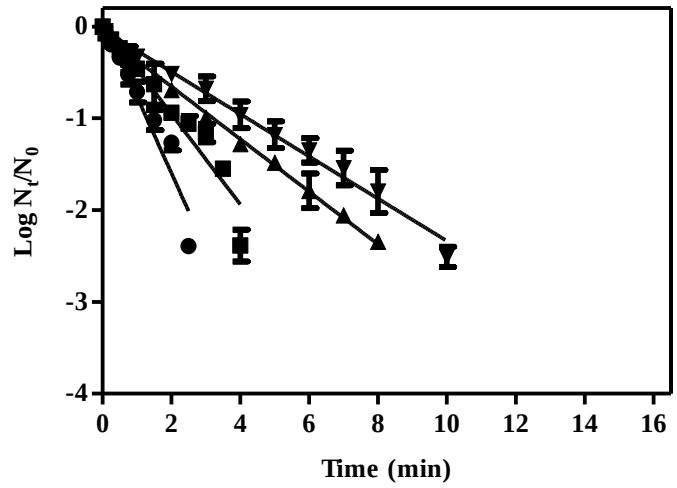

Figure 3.24. Survival curves of *L. monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice at 200 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

Table 3.6. Resistance parameters (D_{MS}) of *L. monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice.

Microorganism	Pressure (kPa)	Ampitude (μm)	Fit Parameters			
			D_{MS} mean (min)	$SD(\pm)$	R^2	Significant Level
L. monocytogenes STCC 5672	0	46.5	11.34	0.15	0.96	$p \leq 0.05$
		90	6.34	0.18	0.98	
		110	4.25	0.03	0.98	
		130.5	4.41	0.40	0.99	
	100	46.5	6.09	0.15	0.99	$p \leq 0.05$
		90	4.01	0.30	0.99	
		110	3.05	0.18	0.99	
		130.5	1.49	0.08	0.98	
	200	46.5	4.41	0.71	0.99	$p \leq 0.05$
		90	3.40	0.16	0.99	
		110	1.87	0.08	0.99	
		130.5	1.05	0.04	0.99	

The variation of log D_{MS} values with amplitude are plotted in Figure 3.25.In addition, $\log_{10}$ number of survivals *vs.* treatment time for effect of amplitude of ultrasonic waves on inactivation rate of *E. coli* O157:H7 for orange juice are given in Figures 3.26, 3.27, and 3.28. Similar to *L. monocytogenes*, MS lethality of *E. coli* decreased because of increase in the amplitude. In most of studies containing microbial inactivation by ultrasonication, similar ultrasonic amplitude effect on lethality have been observed (Raso et al., 1998a; Pagán et al., 1999a, 1999b; Mañas, et al., 2000b; Guerrero et al., 2001; López-Malo et al., 2005; Ugarte-Romero et al., 2007; Arroyo et al., 2011a). For instance, Arroyo et al. (2011a) studied with *C. sakazakii* to evaluate the effect of amplitude (from 34 to 145 μm) on the MS (200 kPa, 35 °C) lethality in buffer (pH 4.0 and 7.0) and apple juice and mentioned that the amplitude was effective parameter on the lethality. Similarly in apple juice, the D_{MS} values decreased exponentially and the MS lethality on *E. coli* increased by 65 to 69 and to 73 % in orange juice at 0,100 and 200 kPa, respectively, when the ultrasonic wave amplitude increased. Additionally, significant difference ($p \leq 0.05$) was found among the magnitude of ultrasound amplitude on MS lethality (App B). Besides, different amplitude-pressure combinations have approximately same effect on MS inactivation rate of *E. coli* O157:H7. As an illustration, the D_{MS} values of 200 kPa pressure-90 μm amplitude combination (D_{MS}= 1.50 min) and 100 kPa pressure- 110 and 130.5 μm amplitudes combinations (D_{MS}= 1.71 and 1.32 min) are nearly equal. In addition, 100 kPa pressure - 90 μm amplitude and 0 kPa pressure - 110 and 130.5 μm amplitudes combinations have almost same D_{MS} values, 2.38, 2.79 and 2.61 min, respectively. In addition, same D_{MS} values are observed in the combinations of 100 kPa-46.5 μm (D_{MS}=4.28 min) and 0 kPa- 90 μm (D_{MS}=4.51 min).

For both microorganisms, using 46.5 μm of amplitude was less effective than 90, 110 and 130.5 μm amplitudes on the inactivation rate in both fruit juices at the selected range of gauge pressure (0,100 and 200 kPa) (Figures 3.14 to 3.28) . When D_{MS} values of 46.5 μm were compared to other amplitudes (Table 3.4 and 3.5), it was obviously seen that there is no need to use this amplitude because of long exposure time for inactivation and less effective inactivation on microorganisms.

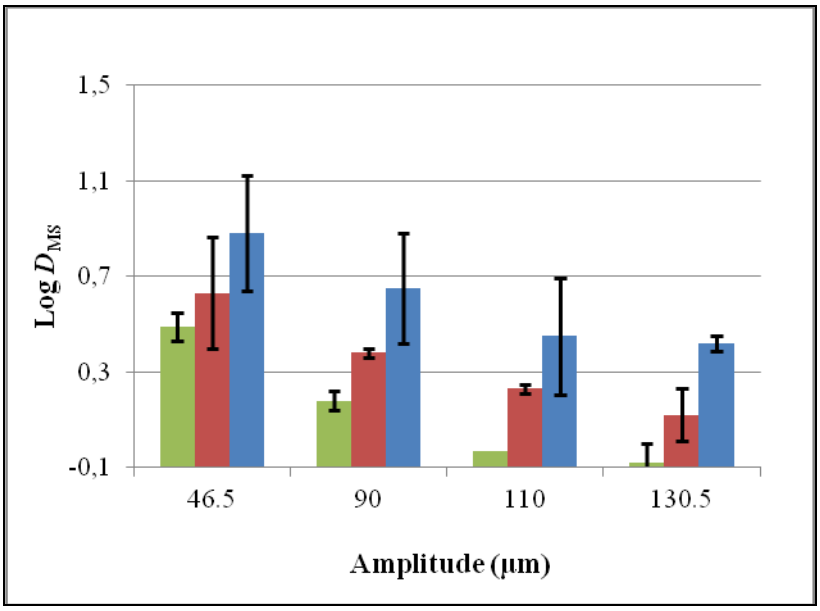

Figure 3.25. Effect of amplitude of US waves (35° C, 20 kHz) on the inactivation rate (Log D_{MS}) of *E. coli* O157:H7 under different pressures [0 kPa (blue bar), 100 kPa (red bar), and 200 kPa (green bar)] in orange juice.

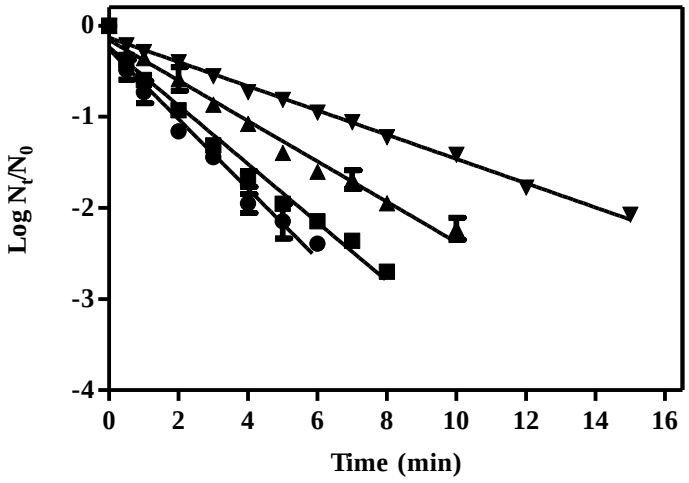

Figure 3.26. Survival curves of *E. coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice at 0 kPa at different amplitudes [46.5 µm (▼), 90 µm (▲), 110 µm (■) and 130.5 µm (●)].

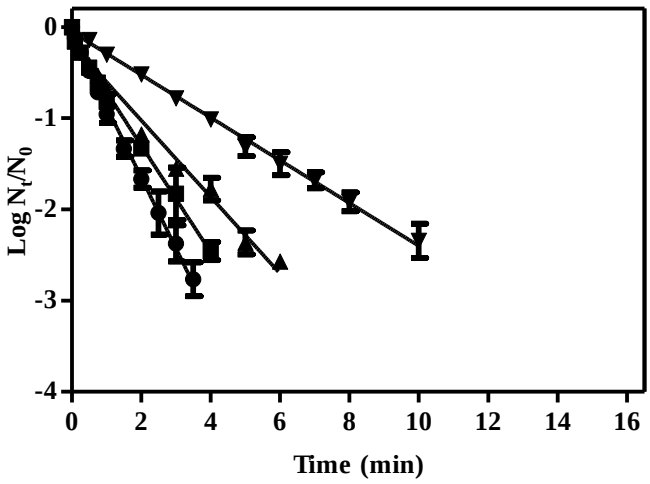

Figure 3.27. Survival curves of *E. coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice at 100 kPa at different amplitudes [46.5 μm (▼), 90 μm (▲), 110 μm (■) and 130.5 μm (●)].

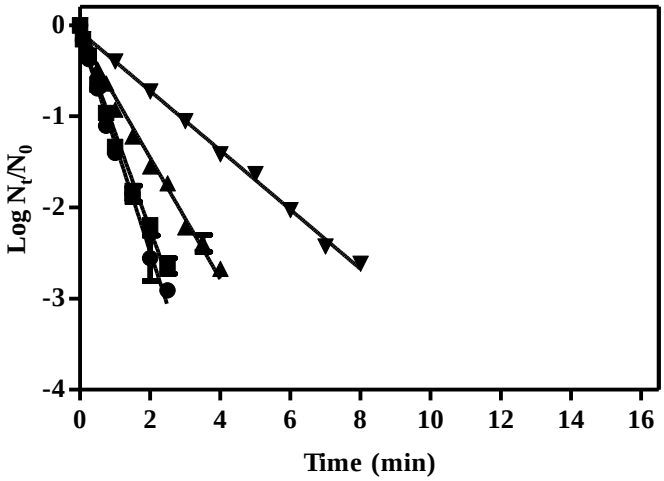

Figure 3.28. Survival curves of *E. coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice at 200 kPa at different amplitudes [46.5 μm (▼), 90 μm (▲), 110 μm (■) and 130.5 μm (●)].

Table 3.7. Resistance parameters (D_{MS}) of *E. coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice.

Microorganism	Pressure (kPa)	Ampitude (μm)	Fit Parameters			
			D_{MS} mean (min)	$SD(\pm)$	R^2	Significant Level
E. coli O157:H7	0	46.5	7.52	0.24	0.99	$p \leq 0.05$
		90	4.51	0.23	0.98	
		110	2.79	0.45	0.98	
		130.5	2.61	0.03	0.97	
	100	46.5	4.28	0.34	0.99	$p \leq 0.05$
		90	2.38	0.02	0.98	
		110	1.71	0.02	0.98	
		130.5	1.32	0.11	0.99	
	200	46.5	3.07	0.06	0.99	$p \leq 0.05$
		90	1.50	0.04	0.99	
		110	0.93	0.00	0.99	
		130.5	0.82	0.08	0.99	

The effect of ultrasonic wave amplitude on the MS lethality of selected microorganisms were plotted in Figures 3.29, 3.30, 3.31 and 3.32 in apple and orange juices, respectively. D_{MS} values of all species investigated decreased exponentially with the amplitude in the range of 46.5 and 130.5 μm for all the pressure range in apple and orange juices. The lethality of MS treatment was also highly affected by amplitude in apple and orange juices. Moreover, the magnitude of the effect of ultrasonic wave amplitude on the microbial resistance was similar for *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 in both juices.

The amplitude of ultrasonic waves has a major effect on microbial inactivation (Condón, et al., 2005). The effect of the amplitude is not related to a change in the intrinsic resistance of each species, but most likely, to its physical effects (Condón et al., 2011). The energy transmitted to the medium by MS/MTS is an exponential function of the amplitude of ultrasonic waves, which also confirms these connections (Raso et al., 1999; Mañas et al., 2000a; Condón et al., 2005, 2011; Arroyo et al., 2011). As result of very high pressure and temperature (Frizzell, 1988; Harvey and Loomis, 1929; Condón et al., 2011) or of the release of free radicals in the medium (Jacobs and Thornley, 1954; Riesz and Kondo, 1992; Condón et al., 2011) by transient cavitation, ultrasonic bacterial inactivation is observed. Increase of amplitude is responsible for higher implosion of micro bubbles per unit of time in a given volume or for increase in the volume of liquid in which transient cavitation occurs (Suslick, 1990).

Raso et al. (1998b) firstly reported an exponential relationship between the amplitude and the number of survivors of Bacillus subtilis spores. Moreover, an exponential relationship between the decimal reduction time and the amplitude has been demonstrated with *Y. enterocolitica* (Raso et al., 1998a) and *L. monocytogenes* (Pagán et al., 1999a). The logarithm of the D_{MS} values decreases linearly with the ultrasonic wave amplitude (Condón et al., 2011) Therefore, Pagán et al. (1999c) firstly introduced a general equation to evaluate the effect of the amplitude on the *D* values for four bacterial species.

$$\text{Log } D = \text{Log } D_0 - 0.0091\ (A - 62)$$

where D is the decimal reduction time in each ultrasonic treatment, D_0 is the decimal reduction time of treatment at amplitude of 62 μm, and A is the ultrasonic wave amplitude. The equation parameters imply that the inactivation rate of vegetative cells by ultrasound will increase by 10 times when increasing the amplitude of ultrasonic waves by 110 microns. Moreover, Arroyo et al. (2011a) calculated the amplitude linear equations for describing the effect of amplitude of sonic waves on the lethality of MS on *C. sakazaii* in four different media. By the same way, the relationship between the D_{MS} values and the amplitude of the ultrasonic waves in each treatment medium can be described by a linear equation in Table 3.8. Because the lethality of MS treatments of both species increased exponentially with the amplitude in the range between 46.5 and 130.5 μm at all pressures in all of the treatment media. Moreover, there were significant differences between the magnitude of ultrasound amplitude on the MS lethality ($p \leq 0.05$) for both species in both juices, however, no significant differences ($p>0.05$) were found among the slopes of the regression lines for the three pressures for both species in both

juices (Appendix B). Therefore, the effect of the amplitude on MS resistance would be the same regardless of the treatment media and the species investigated.

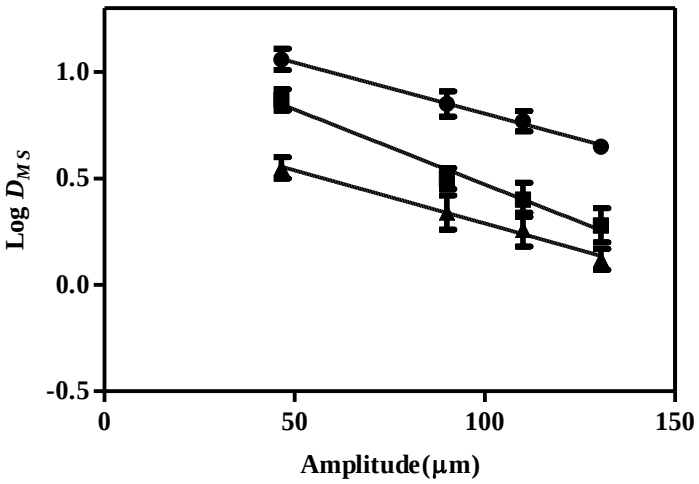

Figure 3.29. Effect of ultrasonic wave amplitude on the resistance of *L. monocytogenes* STCC 5672 in apple juice by MS treatment (35 °C, 20 kHz) under at different pressures [0 kPa (●), 100 kPa (■) and 200 kPa (▲)].

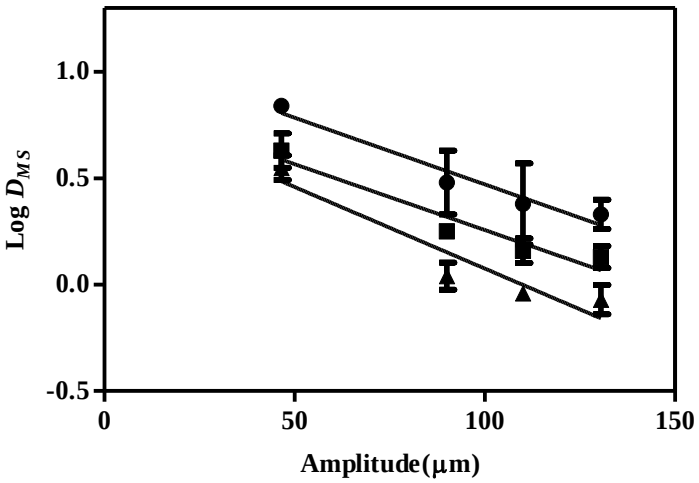

Figure 3.30.Effect of ultrasonic wave amplitude on the resistance of *E.coli* O157:H7 in apple juice by MS treatment (35 °C, 20 kHz) under at different pressures [0 kPa (●), 100 kPa (■) and 200 kPa (▲)].

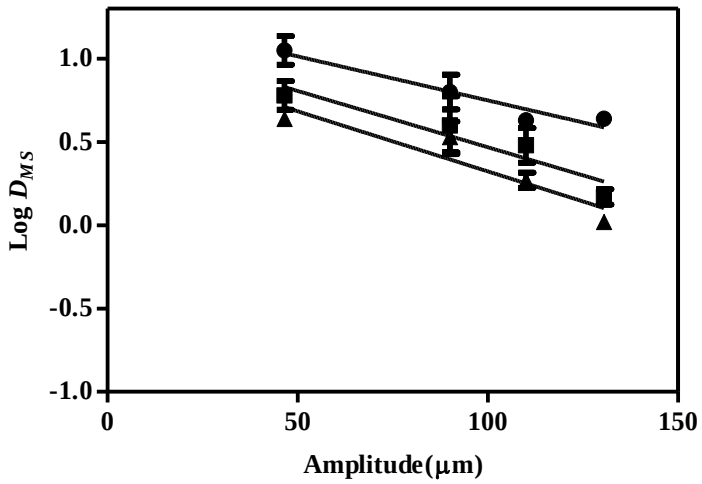

Figure 3.31. Effect of ultrasonic wave amplitude on the resistance of *L. monocytogenes* STCC 5672 in orange juice by MS treatment (35 °C, 20 kHz) under at different pressures [0 kPa (●), 100 kPa (■) and 200 kPa (▲)].

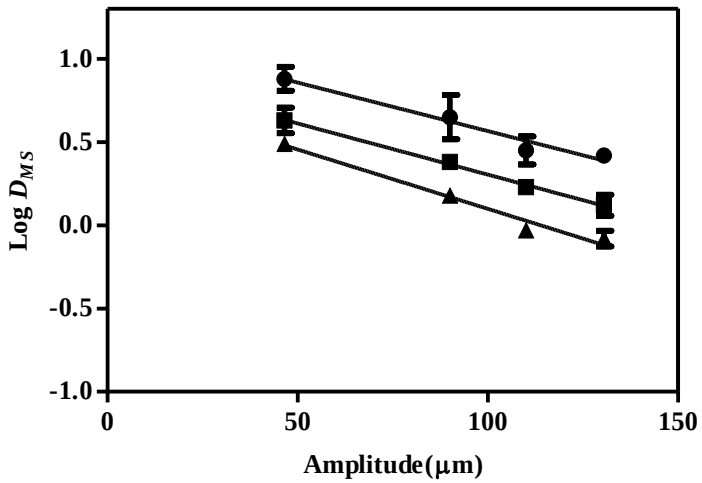

Figure 3.32. Effect of ultrasonic wave amplitude on the resistance of *E. coli* O157:H7 in orange juice by MS treatment (35 °C, 20 kHz) under at different pressures [0 kPa (●), 100 kPa (■) and 200 kPa (▲)].

Table 3.8. Best-fit equations calculated for describing the effect of amplitude of ultrasonic waves on the lethality of MS on *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 treated in apple and orange juices.

Microorganisms	Treatment Media	Pressure (kPa)	Amplitude Linear Equation	R^2
L. monocytogenes STCC 5672	Apple juice	0	Log D_{MS}= - 0.004802 A + 1.285	0.99
		100	Log D_{MS}= - 0.007051 A + 1.177	0.98
		200	Log D_{MS}= - 0.004990 A + 0.788	0.99
	Orange juice	0	Log D_{MS}= - 0.005289 A + 1.278	0.94
		100	Log D_{MS}= - 0.006754 A + 1.144	0.89
		200	Log D_{MS}= - 0.007211 A + 1.045	0.87
E. coli O157:H7	Apple juice	0	Log D_{MS}= -0.006270 A + 1.098	0.95
		100	Log D_{MS}= - 0.006195 A + 0.876	0.93
		200	Log D_{MS}= - 0.007668 A + 0.843	0.90
	Orange juice	0	Log D_{MS}= - 0.005821 A + 1.149	0.96
		100	Log D_{MS}= - 0.006146 A + 0.919	0.99
		200	Log D_{MS}= - 0.007133 A + 0.812	0.98

3.2.5 Effect of Static Pressure on Lethal Effect of MS

The effect of pressure on the inactivation rate of *L. monocytogenes* STCC 5672 according to amplitude for apple and orange juices are plotted in Figures 3.33 to 3.42. Survival curves of *L. monocytogenes* were obtained by plotting the $\log_{10}$ number of survivors *vs.* the treatment time (min) in apple juice (Figures 3.34, 3.35, 3.36, and 3.37) and in orange juice (Figures 3.38, 3.39, 3.40, and 3.41). Figures proved that the D_{MS} values of *L. monocytogenes* in apple and orange juices decreased with the increase in pressure. For instance, the increase in pressure from 0 to 100 kPa reduced D_{MS} values 2.4-fold in apple juice (Table 3.4) and 3-fold in orange juice (Table 3.6) at 130.5 µm constant amplitude. The same increase in pressure (from 100 to 200 kPa) reduced D_{MS} values 1.4-fold in both juices at the same amplitude. The D_{MS} values were reduced 3.4-fold in apple and 4.2-fold in orange juices, when the pressure was increased from 0 to 200 kPa. In both juices, 200 kPa at 130.5 µm amplitude seems to be a more effective condition on the inactivation of *L. monocytogenes*. Other authors in literature (Pagán et al. 1999a, 1999b; Raso et al., 1999; Arroyo et al., 2011a) have reported the similar relationships between the pressure and the MS lethality. Pagán et al. (1999a) mentioned that the increase of pressure in MS treatment (20 kHz, 117 µm, 40 °C) to 200 kPa (from 0 to 200 kPa) decreased D_{MS} to 2.9-fold and increase to 400 kPa (from 200 to 400 kPa) to 1.5-fold. Moreover, the effect of pressure has significant effect ($p \leq 0.05$) on inactivation of *L. monocytogenes* in both fruit juices given in Appendix B.

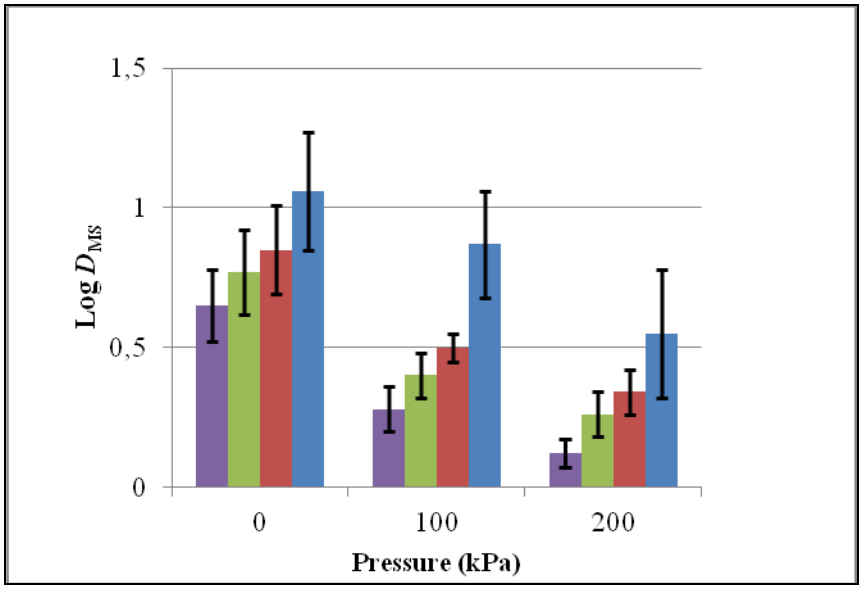

Figure 3.33. Effect of pressure on the MS (35 °C, 20 kHz) inactivation rate (Log D_{MS}) of *L. monocytogenes* STCC 5672 in apple juice at different amplitudes [46.5 µm (blue bar), 90 µm (red bar), 110 µm (green bar) and 130.5 µm (purple bar)].

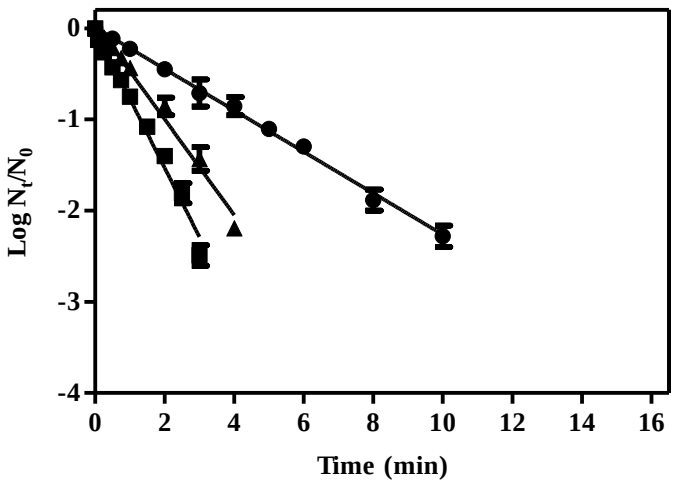

Figure 3.34. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in apple juice at 130.5 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

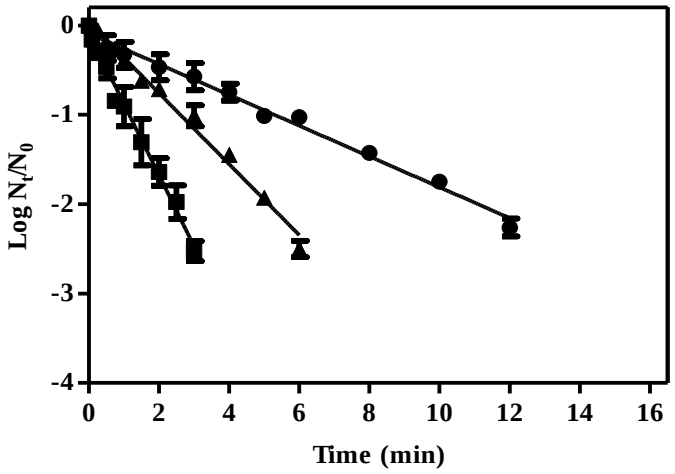

Figure 3.35. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in apple juice at 110 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

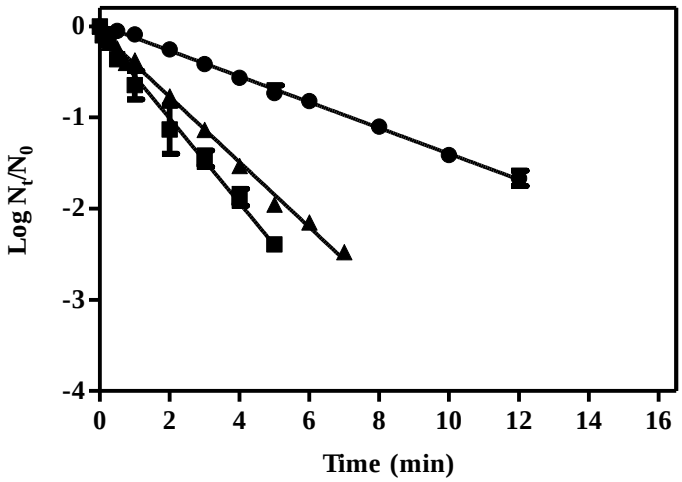

Figure 3.36. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in apple juice at 90 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

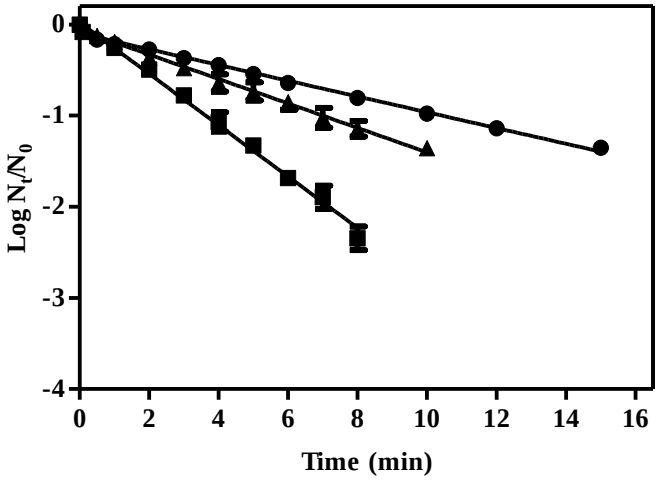

Figure 3.37. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in apple juice at 46.5 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

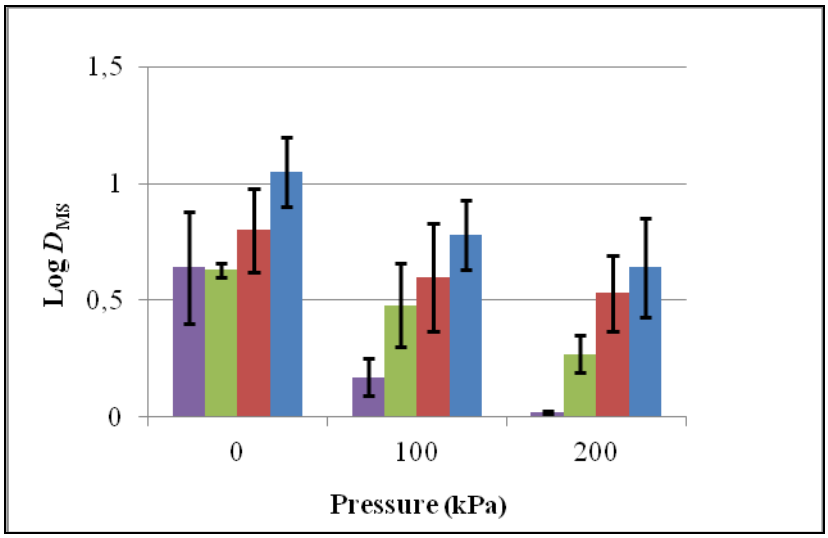

Figure 3.38. Effect of pressure on the MS (35 °C, 20 kHz) inactivation rate (Log D_{MS}) of *L. monocytogenes* STCC 5672 in orange juice at different amplitudes [46.5 µm (blue bar), 90 µm (red bar), 110 µm (green bar) and 130.5 µm (purple bar)].

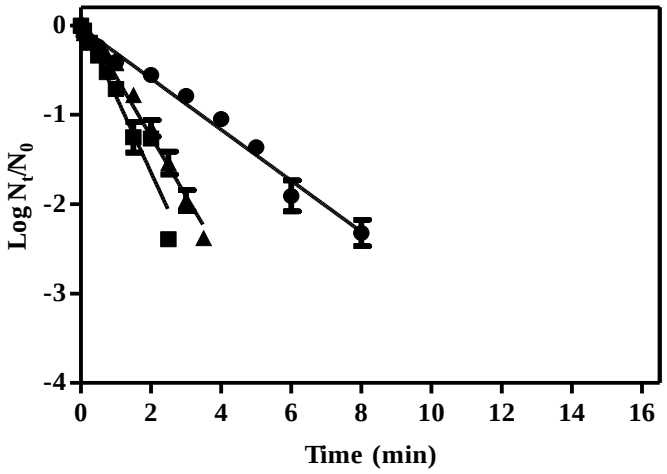

Figure 3.39. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice at 130.5 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

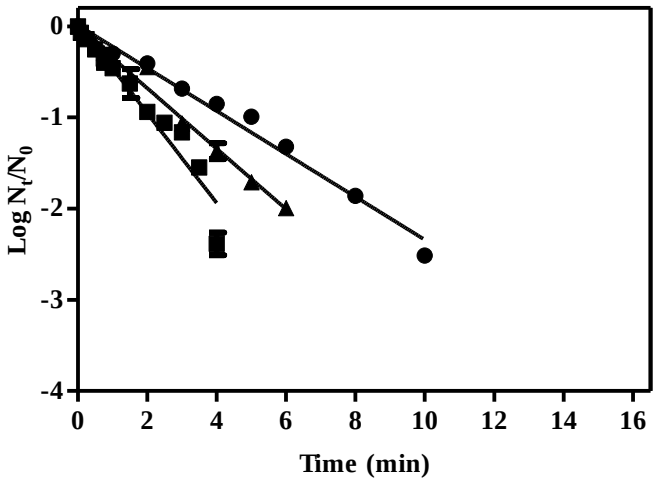

Figure 3.40. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice at 110 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

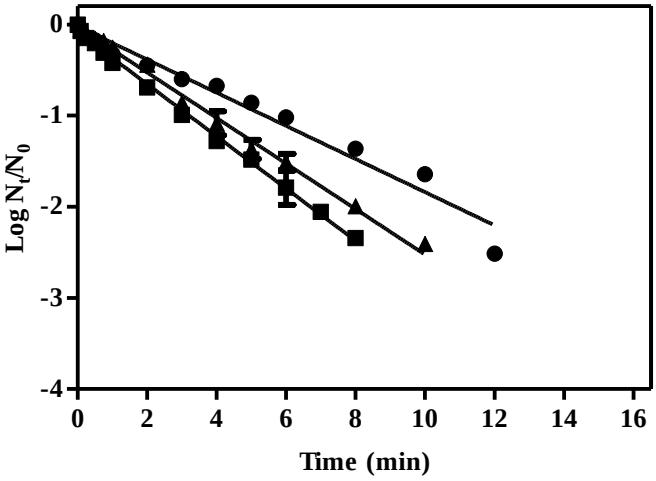

Figure 3.41. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice at 90 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

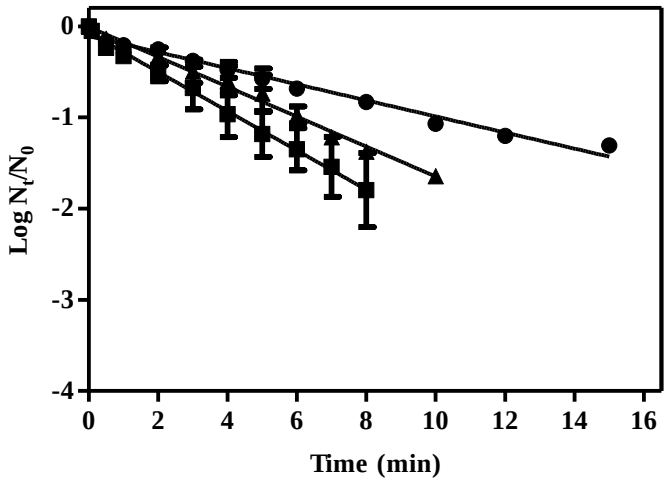

Figure 3.42. Survival curves of *L.monocytogenes* STCC 5672 treated by MS (35 °C, 20 kHz) in orange juice at 46.5 μm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

The effect of pressure on MS inactivation rate of *E. coli* according to amplitude in apple and orange juices are presented in Figures 3.43 to 3.48. The survival curves *vs.* the treatment time (min) data were also plotted at constant amplitudes for apple juice (Figures 3.44, 3.45, 3.46, and 3.47) and orange juice (Figures 3.49, 3.50, 3.51, and 3.52). Similar to *L. monocytogenes* results, these figures proved that increase in pressure increased *E. coli* inactivation in both juices. The increase in pressure from 0 to 100 kPa reduced D_{MS} values 1.6-fold in apple juice (Table 3.5) and 2-fold in orange juice (Table 3.7) at 130.5 μm constant amplitude. In the same way, when pressure was increased from 100 to 200 kPa, D_{MS} values decreased 1.6-fold in both juices at the same amplitude. The D_{MS} values were reduced 2.5-fold in apple and 3.2-fold in orange juices, when the pressure was increased from 0 to 200 kPa. In both juices, 200 kPa at 130.5 μm amplitude proved to be a more effective condition on the inactivation of *E. coli*. Our results were also in agreement with the findings of Pagán et al. (1999a) for *L. monocytogenes*, of Raso et al. (1999a) for *Y. enterecolitica*, and of Arroyo et al. (2011a) for *C. sakazakii* in McIlvaine citrate-phosphate buffer (pH 7.0). In the study of Raso et al. (1999a), the D_{MS} of *Y. enterecolitica* reduced 5.4-fold and 1.4-fold, while the pressure increased from 0 to 300 kPa and from 300 to 600 kPa, respectively, during the MS treatment (20 kHz, 150 μm, 40 °C). Statistical analysis determined the significant differences ($p \leq 0.05$) between the effect of the pressure and the D_{MS} values of inactivation rate of *E. coli* O157:H7 were given in Appendix B.

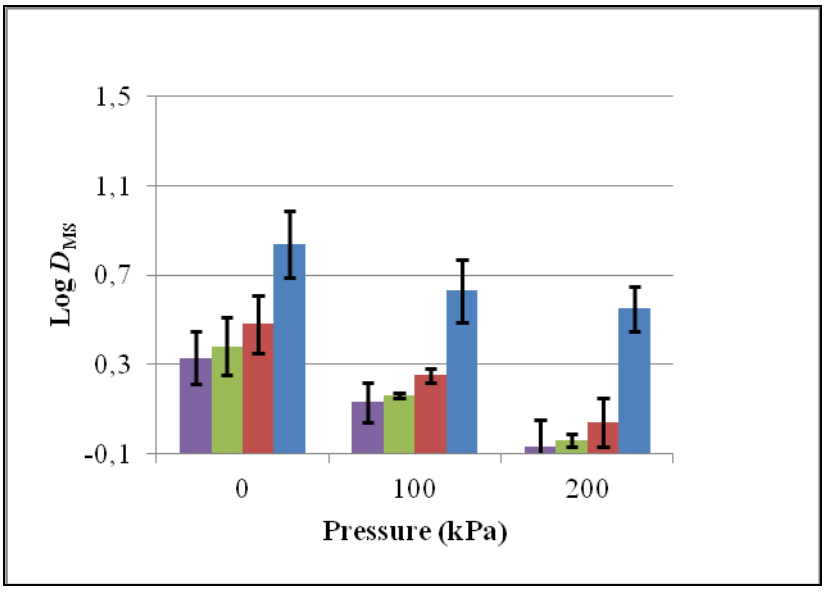

Figure 3.43. Effect of pressure on the MS (35 °C, 20 kHz) inactivation rate (Log D_{MS}) of *E. coli* O157:H7 in apple juice at different amplitudes [46.5 µm (blue bar), 90 µm (red bar), 110 µm (green bar) and 130.5 µm (purple bar)].

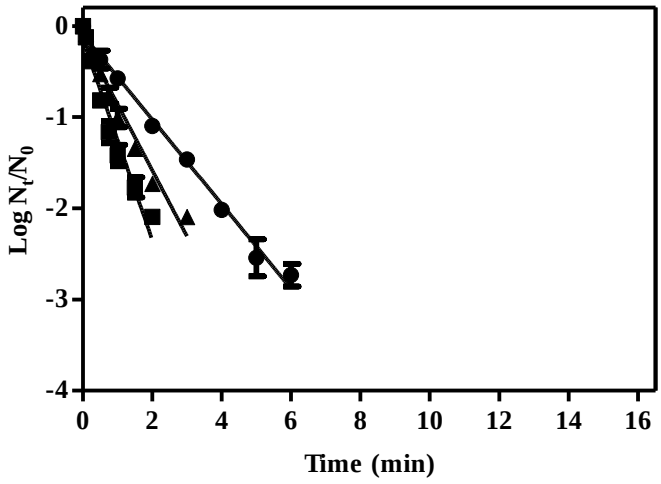

Figure 3.44. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice at 130.5 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

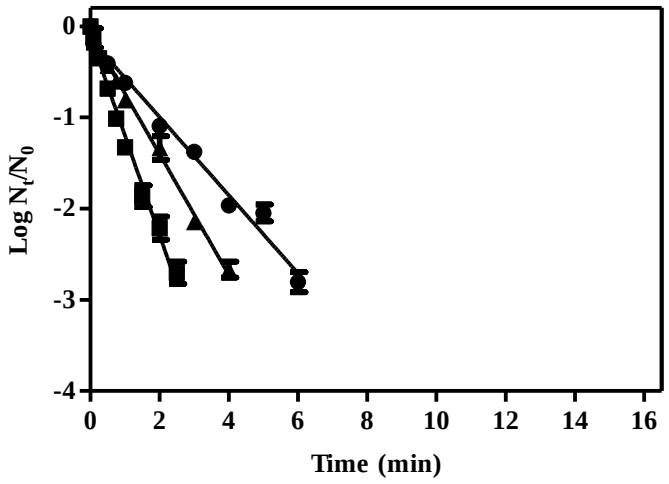

Figure 3.45. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice at 110 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

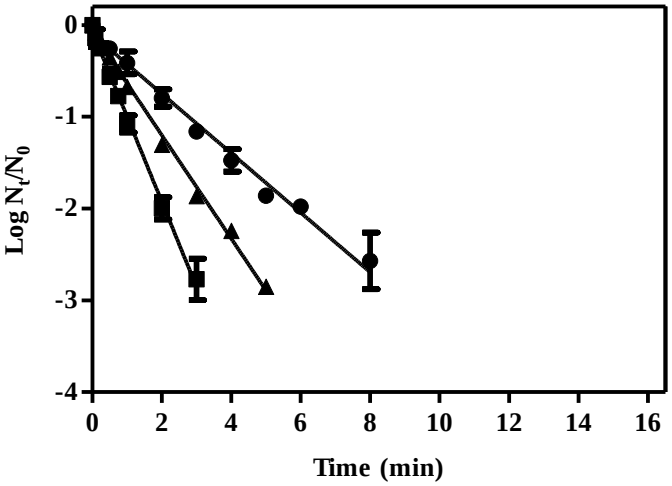

Figure 3.46. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice at 90 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

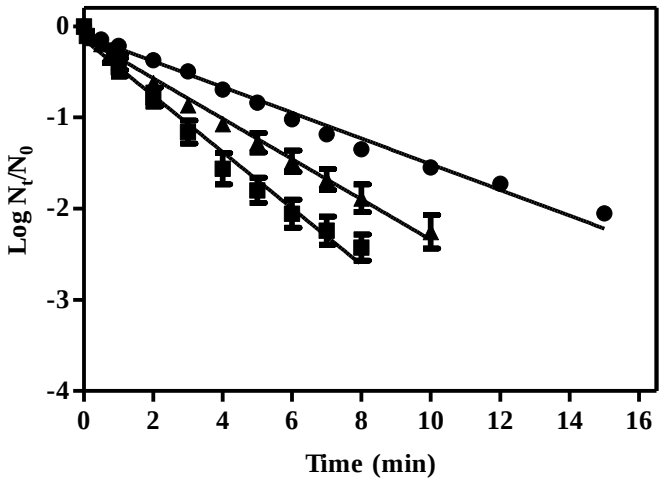

Figure 3.47. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in apple juice at 46.5 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

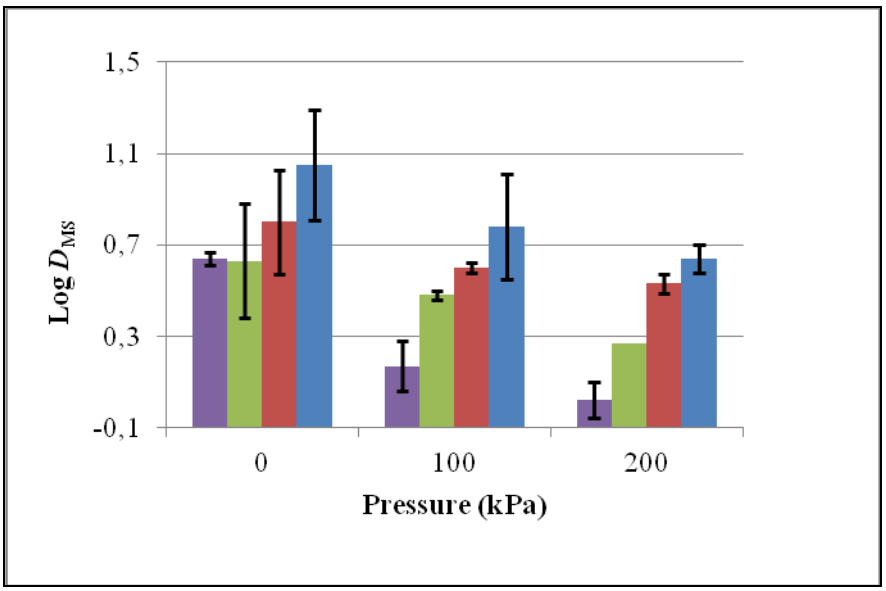

Figure 3.48. Effect of pressure on the MS (35 °C, 20 kHz) inactivation rate (Log D_{MS}) of *E. coli* O157:H7 in orange juice at different amplitudes [46.5 µm (blue bar), 90 µm (red bar), 110 µm (green bar) and 130.5 µm (purple bar)].

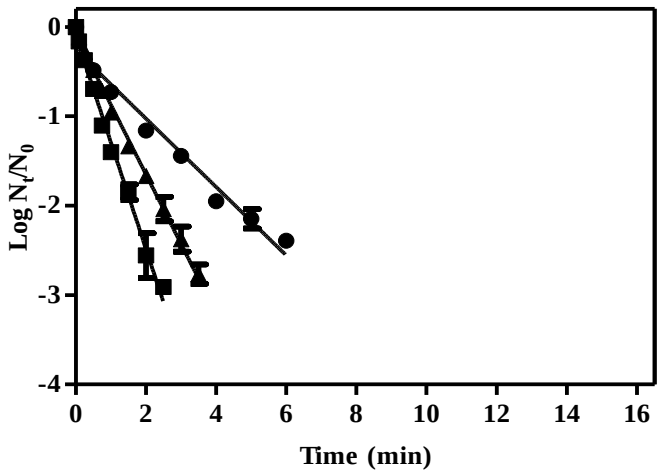

Figure 3.49. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice at 130.5 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

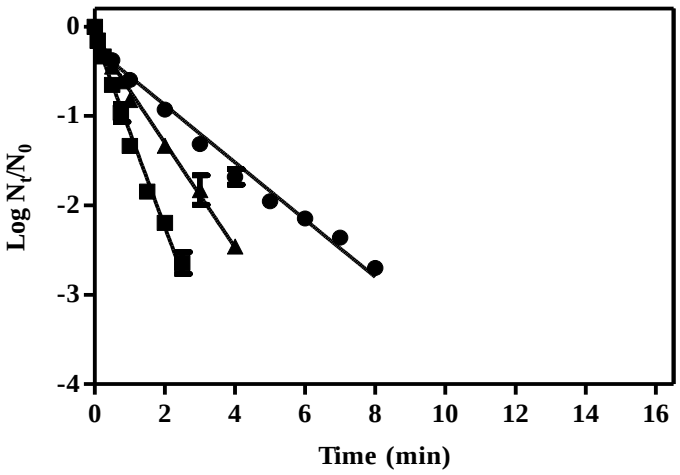

Figure 3.50. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice at 110 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

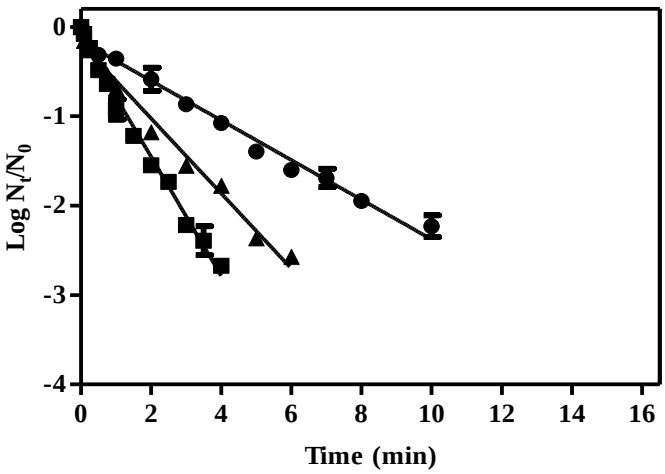

Figure 3.51. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice at 90 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

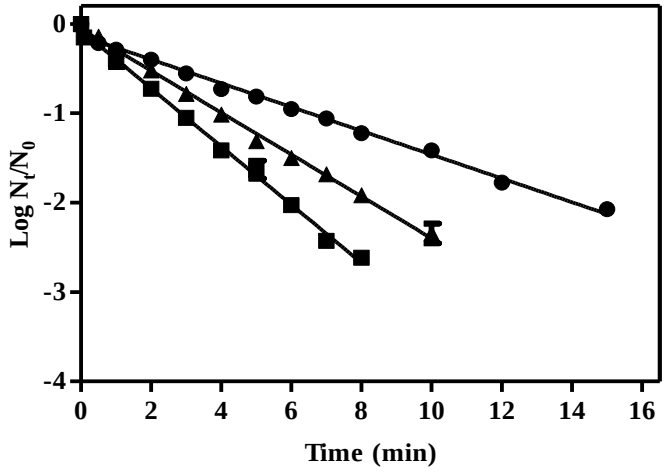

Figure 3.52. Survival curves of *E.coli* O157:H7 treated by MS (35 °C, 20 kHz) in orange juice at 46.5 µm amplitude at different pressures [0 kPa (●), 100 kPa (▲) and 200 kPa (■)].

The inactivation rates of both microorganisms by ultrasonic treatments at sub lethal temperatures (up to 40 °C) increased exponentially by raising the static relative pressure (MS treatment) in apple and orange juices (Figures 3.57, 3.58, 3.59 and 3.60). In all cases, the rise in lethality became progressively smaller as the pressure increased. Suslick (1988) and Whillock and Harvey (1997) stated that pressure increases the cavitation intensity of ultrasound. A good fit of a unique equation to the experimental data obtained for different pressures with different species, indicated that the differences in cell structure do not affect the effect of pressure, which will most likely be relevant to changes in cavitation intensity (Whillock and Harvey, 1997; Condón et al., 2011). During US treatment, when the hydrostatic pressure of a system increases, the intramolecular cohesion forces are strengthened, that reduces the tension of the medium vapor and increases medium viscosity. These both circumstances prevent the transient cavitation phenomenon. However, if power supply produces sufficient energy to sustain the amplitude of the ultrasonic waves and cavitation occurs, its physicochemical and biological effects are higher (Berliner, 1984). Therefore, the increase in pressure probably caused higher lethal effects due to an increasing of micro bubbles implosion (Suslick, 1988; Whillock and Harvey, 1997; Condón et al., 2011). At the same time, the reason of the lower response to increasing pressure at high level (above 400 kPa) was due to reducing of the number of bubbles sustained cavitation (Suslick,1988), as well as the microbial inactivating effect of the treatment (Condón et al., 2011). This means an increased pressure not always will lead to increased lethal efficacy (Condón et al., 2011). Raso et al. (1998a) also found that there were no significant differences between D_{MS} values (20 kHz, 40 °C, 117 μm) obtained 400 and 600 kPa for *Y. enterocolitica* in McIlvaine citrate-phosphate buffer pH 7.0.

To explain the relationship between pressure and MS inactivation, other authors have proposed a quadratic equation (Raso et al., 1998a, 1998b; Pagán et al.,1999a, 1999b; Mañas et al., 2000a; Arroyo et al., 2011a); however, in the small range of pressure (0-200 kPa), an exponential function adequately described the relationship. The relationship between pressure and MS inactivation rate can be described by linear equation in Table 3.9. This equation calculation allows the adjustment or prediction of all D_{MS} values of vegetative cells at sub lethal temperature and at different pressures. Moreover, there were significant differences between the magnitude of pressure on the MS lethality ($p \leq 0.05$) for both species in both juices, however, no statistically significant differences ($p>0.05$) were found among the slopes of the regression lines for the selected pressures for both species in fruit juices (App. B). Thus, the effect of the pressure on MS resistance would be the same regardless of the treatment media and the species investigated. These results coincide with the finding of Raso et al. (1998a, 1998b); Pagán et al. (1999a, 1999b, 1999c); Mañas et al. (2000a); Guerrero et al. (2001); López-Malo et al. (2005); Adekunte et al. (2010b); and Arroyo et al. (2011a) and it can be concluded that the contribution of ultrasound to inactivation increased as the pressure is increased.

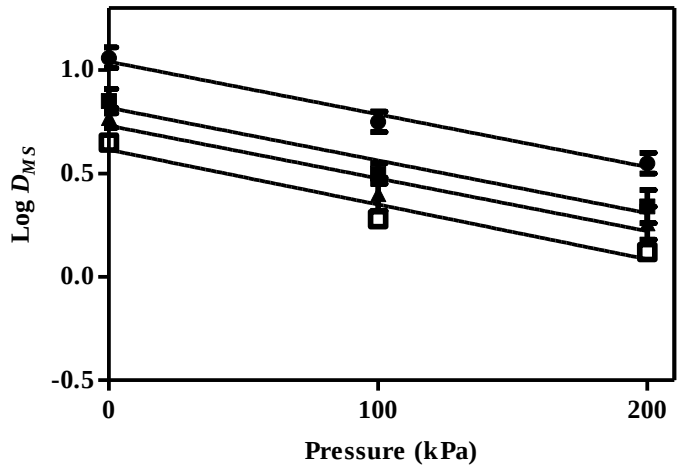

Figure 3.53. Effect of static pressure on the resistance of *L. monocytogenes* STCC 5672 in apple juice by MS treatment (35° C, 20 kHz) at different amplitudes [46.5 µm (●),90 µm (■), 110 µm (▲) and 130.5 (□)].

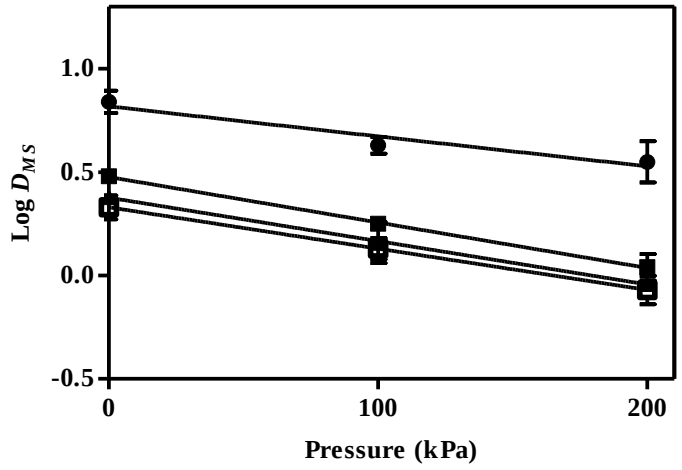

Figure 3.54.Effect of static pressure on the resistance of *E. coli* O157:H7 in apple juice by MS treatment (35° C, 20 kHz) at different amplitudes [46.5 µm (●), 90 µm (■), 110 µm (▲) and 130.5 (□)].

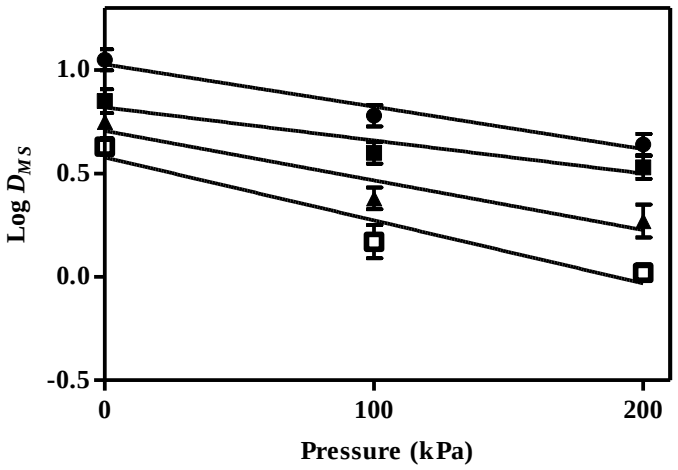

Figure 3.55. Effect of static pressure on the resistance of *L. monocytogenes* STCC 5672 in orange juice by MS treatment (35° C, 20 kHz) at different amplitudes [46.5 µm (●), 90 µm (■), 110 µm (▲) and 130.5 (□)].

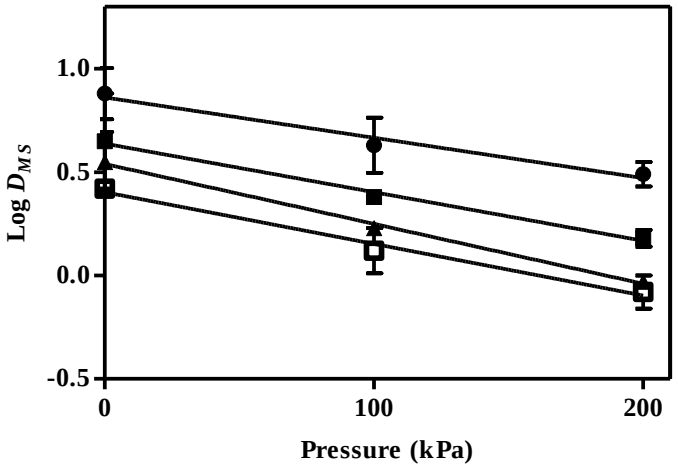

Figure 3.56. Effect of static pressure on the resistance of *E. coli* O157:H7 in orange juice by MS treatment (35° C, 20 kHz) at different amplitudes [46.5 µm (●),90 µm (■), 110 µm (▲) and 130.5 (□)].

Table 3.9. Best fit equations calculated for describing the effect of pressure on the lethality of MS on *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 treated in apple and orange juices.

Microorganisms	Treatment Media	Ampitude (μm)	Pressure Linear Equation	R^2
L. monocytogenes STCC 5672	Apple juice	46.5	Log D_{MS}= - 0.00255 P + 1.042	0.98
		90	Log D_{MS}= - 0.00255 P + 0.818	0.96
		110	Log D_{MS}= - 0.00255 P + 0.732	0.94
		130.5	Log D_{MS}= - 0.00265 P + 0.615	0.95
	Orange juice	46.5	Log D_{MS}= - 0.00205 P + 1.028	0.97
		90	Log D_{MS}= - 0.00160 P + 0.820	0.91
		110	Log D_{MS}= - 0.00240 P + 0.707	0.91
		130.5	Log D_{MS}= - 0.00350 P + 0.578	0.92
E. coli O157:H7	Apple juice	46.5	Log D_{MS}= - 0.00145 P + 0.818	0.94
		90	Log D_{MS}= - 0.00220 P + 0.477	0.99
		110	Log D_{MS}= - 0.00210 P + 0.377	0.99
		130.5	Log D_{MS}= - 0.00200 P + 0.330	0.97
	Orange juice	46.5	Log D_{MS}= - 0.00195 P + 0.862	0.97
		90	Log D_{MS}= - 0.00235 P + 0.638	0.99
		110	Log D_{MS}= - 0.00290 P + 0.540	0.99
		130.5	Log D_{MS}= - 0.00250 P + 0.403	0.99

3.2.6 Thermal Treatment

The survival curves of *L. monocytogenes* and *E. coli* at different temperatures (50, 55 and 60°C) in apple and orange juices are given in Figures 3.57, 3.58, 3.59 and 3.60. Regarding the kinetics of inactivation, while survival curves obtained after MS treatment were linear ($R^2 \geq 0.97$) as previously stated those obtained after TT showed a downward concavity (Figures 3.57 to 3.60). Therefore, survival curves were fitted with Weibull model with Mafart equation (Eq.2.1) to obtain the resistance parameters (δ and ρ values) and thus, to estimate the time to achieve a certain degree of inactivation (1*D* or 4*D*). Some researchers (Peleg et al., 2000; Mafart et al., 2002; Arroyo et al., 2009) have reported the occurrence of downward concavity in the survival curves to TT. Moreover, it is noteworthy to mention that the concave downward profiles only appeared when apple and orange juices were used as treatment media, but not when buffer of the same pH was used as treatment media. Microbial thermal resistance is usually higher in foods than in buffers (Hansen and Riemann, 1963; Moats et al., 1971; Mañas et al., 2001) and as foods are very complex chemical systems, in which most components (salts, sugars, protein, fats, etc.) may affect microbial thermal tolerance. Arroyo et al. (2009) also demonstrated that *E. sakazakii* proved to be more heat resistant in four different liquid food matrixes (Orange juice, milk powder, vegetable and chicken soups) than in buffers at the same pH. As can be seen from the figures, D_{TT} values decreased with temperature. From the exponential relationship between Log D_{TT} values and temperature, z_{TT} values can be calculated for selected microorganisms in both fruit juices (Table 3.10). When comparing the D_{TT} values of TT inactivation of both species, significant differences were found ($p \leq 0.05$; App. B).

In contrast to the interspecific strain behavior shown under MS treatments, it should be noticed that the Gram-negative *E. coli* O157:H7 displayed a higher thermal resistant than the Gram-positive *L. monocytogenes* STCC 5672 in both fruit juices (Table 3.10). As can be seen in Figure 3.65, at 50 °C, D_{TT} values of *E. coli* O157:H7 are 6.5-fold and 6-fold higher than D_{TT} values of *L. monocytogenes* STCC 5672 in apple and orange juices, respectively. Moreover, for *L. monocytogenes*, D_{TT} value at 50 °C is 2.7-fold and 11-fold higher than D_{TT} values at 55 and 60 °C in apple juice, respectively. At same conditions in orange juice, D_{TT} values at 50°C of *E. coli* are 4.3-fold and 49-fold higher than D_{TT} values at 55 and 60 °C, respectively (Figure 3.65). These results would point out *E. coli* O157:H7 instead of *L. monocytogenes* STCC 5672 as a target microorganism when processing fruit juices by TT.

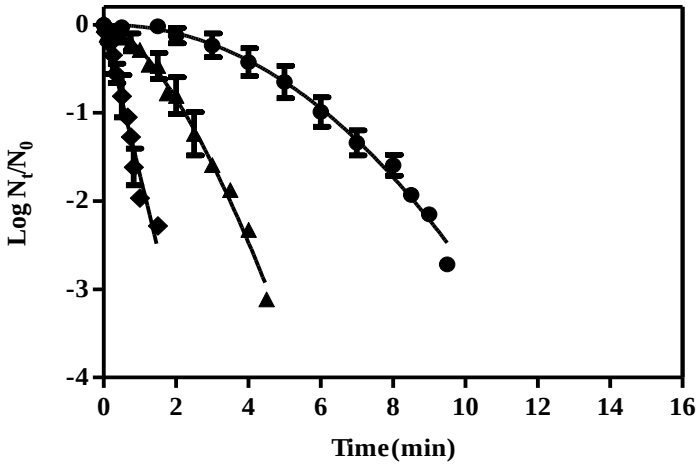

Figure 3.57. Survival curves of *L. monocytogenes* STCC 5672 in apple juice treated by TT (200 kPa) at different temperatures: 50 °C (●), 55 °C (▲) and 60 °C (♦).

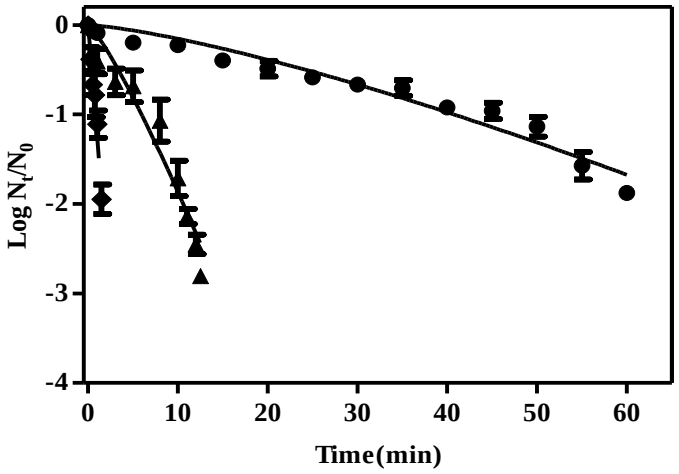

Figure 3.58. Survival curves of *E. coli* O157:H7 in apple juice treated by TT (200 kPa) at different temperatures: 50 °C (●), 55 °C (▲) and 60 °C (♦).

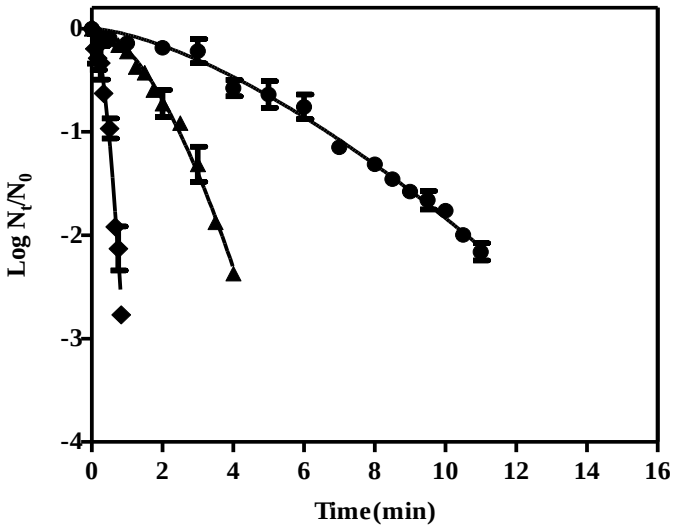

Figure 3.59. Survival curves of *L. monocytogenes* STCC 5672 in orange juice treated by TT (200 kPa) at different temperatures: 50 °C (●), 55 °C (▲) and 60 °C (♦).

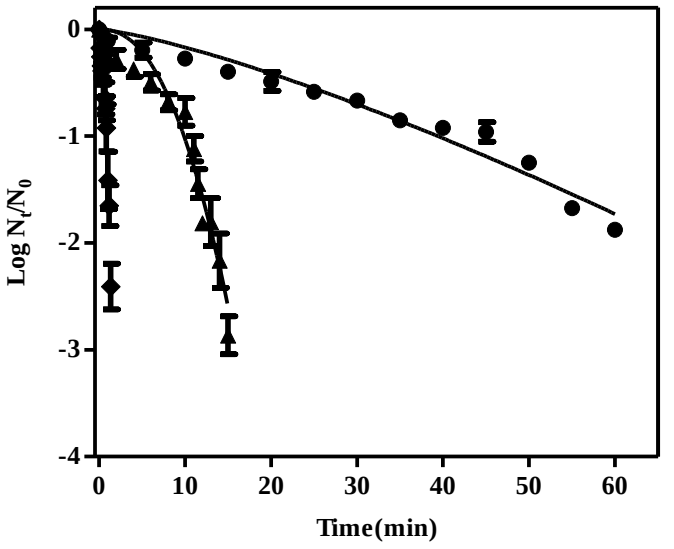

Figure 3.60. Survival curves of *E. coli* O157:H7 in orange juice treated by TT (200 kPa) at different temperatures: 50 °C (●), 55 °C (▲) and 60 °C (♦).

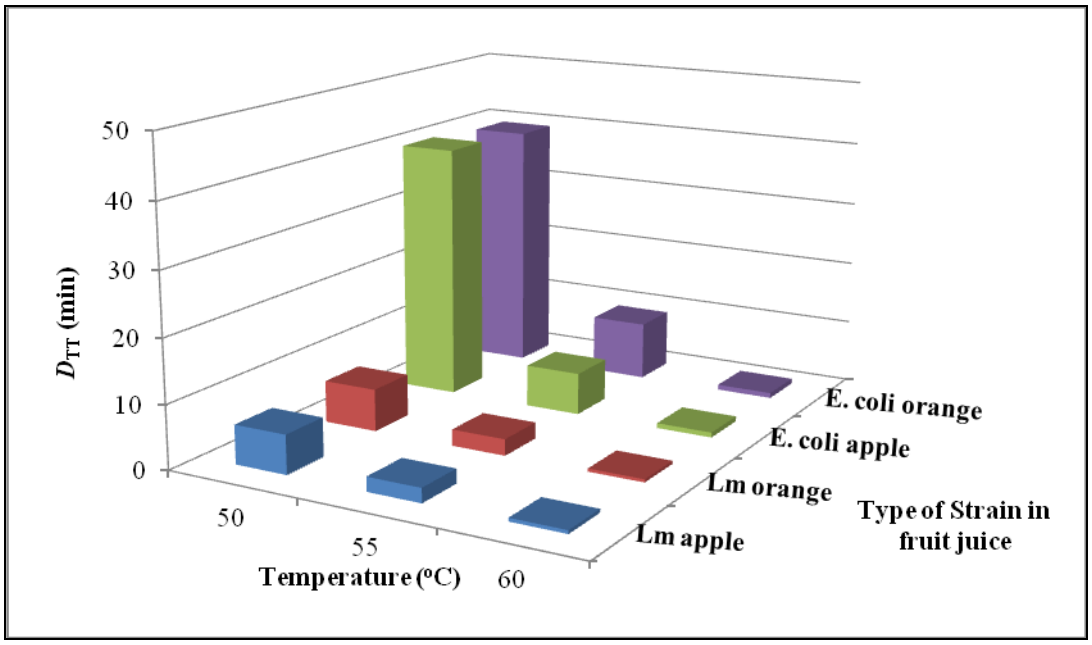

Figure 3.61. Thermal resistances of *L. monocytogenes* STCC 5672 and *E. coli* O157: H7 in apple and orange juices at different temperatures: 50, 55 and 60 °C.

Table 3.10. Resistance parameters (D_{TT}) of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 to TT in apple and orange juices.

Microorganisms	Treatment media	pH	Pressure (kPa)	Temperature (^{o}C)	Fit Parameters			
					D_{TT} mean (min)	$SD(\pm)$	R^2	Significant Level
L. monocytogenes STCC 5672	Apple juice	3.4	200	50	6.158	0.526	0.97	$p \leq 0.05$
				55	2.287	0.294	0.98	
				60	0.552	0.062	0.98	
	Orange juice	3.7	200	50	6.657	0.366	0.99	$p \leq 0.05$
				55	2.582	0.123	0.98	
				60	0.482	0.030	0.98	
E. coli O157:H7	Apple juice	3.4	200	50	40.167	0.955	0.94	$p \leq 0.05$
				55	6.630	1.011	0.92	
				60	0.783	0.071	0.94	
	Orange juice	3.7	200	50	39.377	0.950	0.96	$p \leq 0.05$
				55	9.167	0.319	0.93	
				60	0.811	0.066	0.90	

3.2.7 MTS Experiments

3.2.7.1 Effect of Temperature on Lethal Effect of MTS

Although different studies are available in the literature studying the lethal effect of ultrasound combined with heat in different media such as buffer (Pagán et al., 1999a; Mañas et al., 2000b; Zenker et al., 2003; Lee et al., 2009b; Arroyo et al., 2011b), milk (Pagán et al., 1999a; Villamiel & Jong, 2000a; Zenker et al, 2003; Arroyo et al., 2011b), orange juice (Zenker et al., 2003), carrot juice (Zenker et al., 2003), apple juice (Arroyo et al., 2012), distilled water (Cabeza et al., 2004), broth (Guerrero et al., 2001; López-Malo et al., 2005), and liquid whole egg (Mañas et al., 2000b), it is needed to concern the effect of treatment temperature on microbial ultrasonic resistance in acidic food products. Therefore, in this work, the inactivation of *L. monocytogenes* and *E. coli* by MTS at different temperatures (50, 55, and 60 °C) in apple and orange juices was studied. Figures 3.62, 3.63, 3.64 and 3.65 show the effect of temperature on survival curves of *L. monocytogenes* STCC 5672 by ultrasound under pressure at constant amplitude. For *E. coli* O157:H7, the effect of temperature on survival curves was plotted in Figures 3.66, 3.67, 3.68, and 3.69. Although shoulders (Adekunte et al., 2010a, 2010b), tails (Sala et al., 1995; Lee et al., 2009b) and sigmoid profiles (Bermudez-Aguirre et al., 2009) have been found by some researchers, a first-order kinetic was observed in most of ultrasound technology studies related to microbial inactivation (Kinsloe et al., 1954; Davies, 1959; Sala et al., 1995; Pagán et al., 1999a, 1999b; Lee et al., 2009b; Condón et al., 2011). This linear shape in MTS survival curves was also found when *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 were treated at different temperatures.

Changes in the lethal efficacy of ultrasound at different temperatures may be associated to the direct and indirect mechanisms. The increasing temperature of the medium may cause not only lose a part of mechanical resistance of vegetative cells, but also some structural changes in those cells. The intensity of cavitation affected the temperature of the liquid under sonication experiments. The formation and growth of bubbles consist of rapidly as the temperature of the liquid medium increases, because the vapor pressure also increases and tensile strength decreases (Suslick, 1988). On the other hand, because of high vapor tensile inside the bubble as known a cushion effect, the violence of bubble collapse is lower (Alliger, 1975) and the effect of cavitation in the treatment medium decreases. Therefore, the final biological effect of the combine treatment of heat and US will depend on the balance between the direct and indirect mechanisms, whose relative significant relationship will also depend on the range of temperatures under study (Condón et al., 2011).

As can be seen from Figures 3.62 to 3.69, there are significant differences between the inactivation lines of *L. monocytogenes* and *E. coli* in both treatment media ($p \leq 0.05$). When the D_{MTS} values of inactivation are investigated in Table 3.11, for same treatment media, the D_{MTS} values are close each other at same temperature and pressure applications for both species.

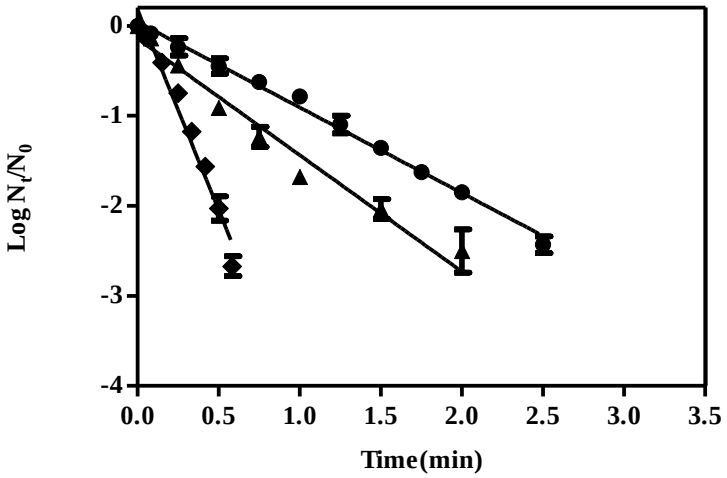

Figure 3.62. Survival curves of *L. monocytogenes* STCC 5672 in apple juice by ultrasound (20 kHz, 110 μm) under constant pressure (200 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

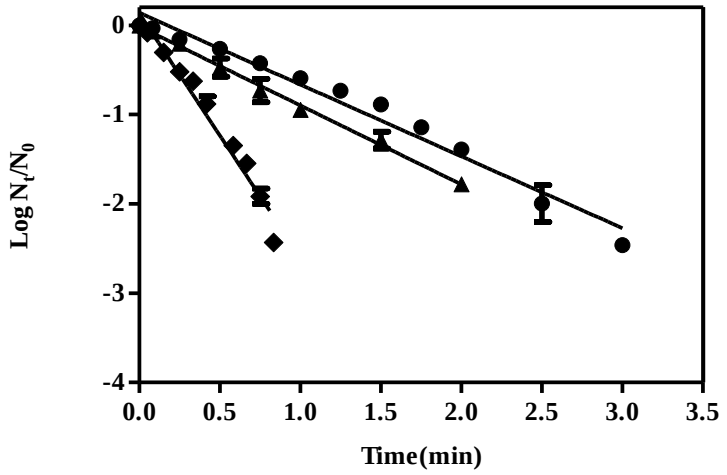

Figure 3.63. Survival curves of *L. monocytogenes* STCC 5672 in apple juice by ultrasound (20 kHz, 110 μm) under constant pressure (100 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

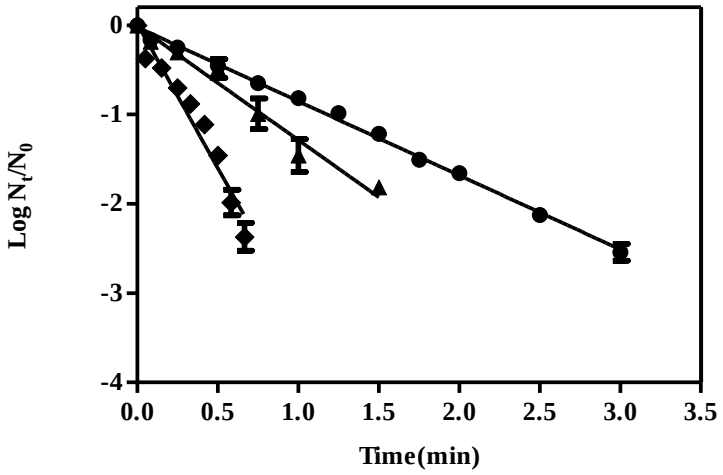

Figure 3.64. Survival curves of *L. monocytogenes* STCC 5672 in orange juice by ultrasound (20 kHz, 110 µm) under constant pressure (200 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

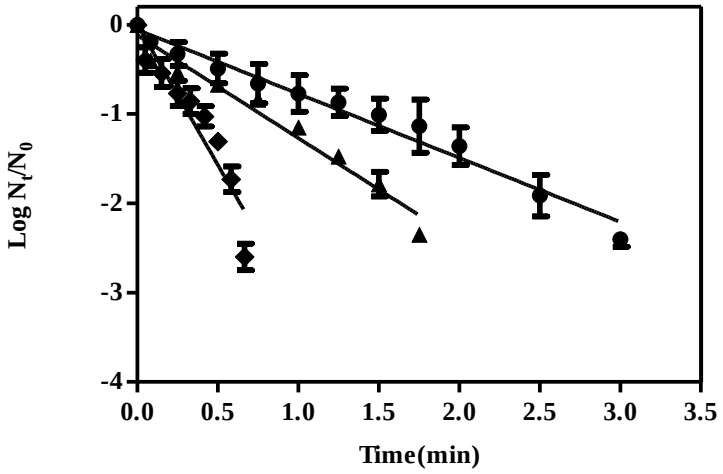

Figure 3.65. Survival curves of *L. monocytogenes* STCC 5672 in orange juice by ultrasound (20 kHz, 110 µm) under constant pressure (100 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

Table 3.11. Resistance parameters (D_{MTS}) of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 to MTS in apple and orange juices.

Microorganisms	Treatment Media	pH	Ampitude (μm)	Pressure (kPa)	Temperature (°C)	Fit Parameters			
						D_{MTS} mean (min)	*SD* (±)	R^2	Significant Level
L. monocytogenes STCC 5672	Apple Juice	3.4	110	100	50	1.24	0.04	0.97	$p \leq 0.05$
					55	1.13	0.07	0.99	
					60	0.37	0.00	0.97	
				200	50	1.05	0.04	0.99	$p \leq 0.05$
					55	0.77	0.05	0.97	
					60	0.23	0.01	0.97	
	Orange Juice	3.7	110	100	50	1.40	0.07	0.97	$p \leq 0.05$
					55	0.87	0.03	0.96	
					60	0.32	0.01	0.90	
				200	50	1.20	0.00	0.99	$p \leq 0.05$
					55	0.76	0.12	0.96	
					60	0.31	0.01	0.95	
E. coli O157:H7	Apple Juice	3.4	110	100	50	1.21	0.16	0.99	$p \leq 0.05$
					55	0.60	0.03	0.98	
					60	0.36	0.01	0.98	
				200	50	0.89	0.08	0.99	$p \leq 0.05$
					55	0.52	0.07	0.98	
					60	0.27	0.02	0.96	
	Orange Juice	3.7	110	100	50	1.15	0.02	0.99	$p \leq 0.05$
					55	0.83	0.01	0.99	
					60	0.34	0.01	0.98	
				200	50	0.77	0.06	0.99	$p \leq 0.05$
					55	0.59	0.01	0.99	
					60	0.29	0.01	0.92	

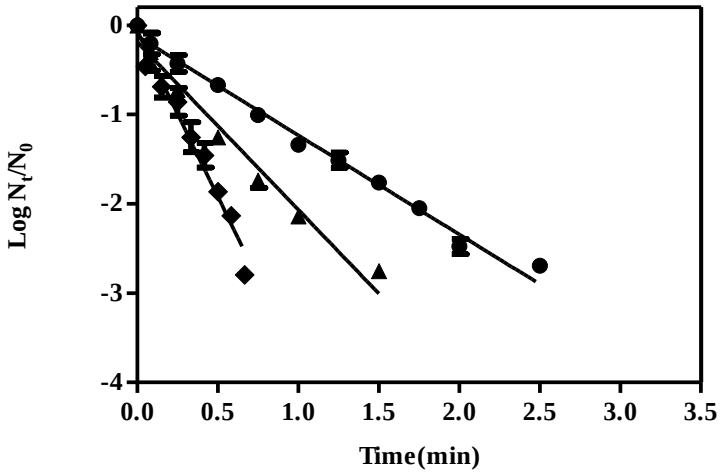

Figure 3.66. Survival curves of *E. coli* O157:H7 in apple juice by ultrasound (20 kHz, 110 µm) under constant pressure (200 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

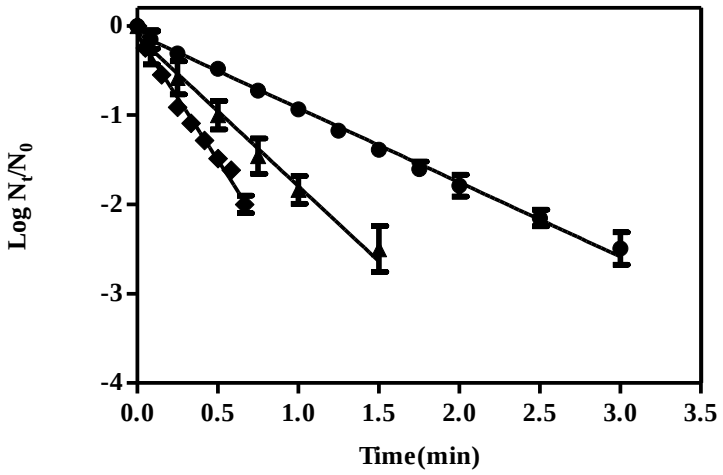

Figure 3.67. Survival curves of *E. coli* O157:H7 in apple juice by ultrasound (20 kHz, 110 µm) under constant pressure (100 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

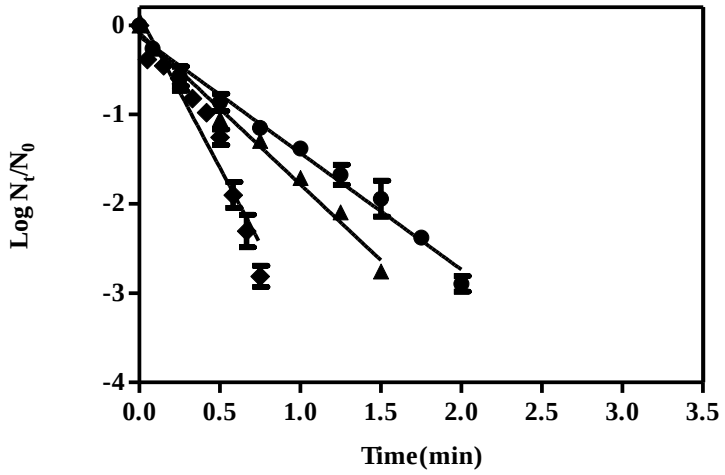

Figure 3.68. Survival curves of *E. coli* O157:H7 in orange juice by ultrasound (20 kHz, 110 µm) under constant pressure (200 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

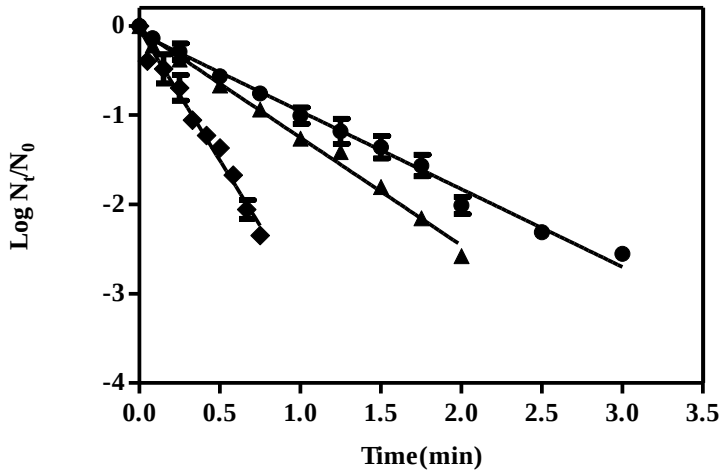

Figure 3.69. Survival curves of *E .coli* O157:H7 in orange juice by ultrasound (20 kHz, 110 µm) under constant pressure (100 kPa) at different temperatures [50°C (●), 55°C (▲) and 60°C (♦)].

Figures 3.70 and 3.71 show the $D_{MS/MTS}$ values of both species *vs.* temperature (35, 50, 55 and 60 °C) at different pressures (100 and 200 kPa) at constant amplitude (110 µm) in both treatment media. Treatment with ultrasound alone in food industry needs longer time for an effective 5-Log inactivation (USFDA, 2000; Sagong et al., 2011). As mentioned previously, because of some morphological features of bacteria (rod or coccus shape, gram negative or positive), $D_{MS/MTS}$ values of *L. monocytogenes* are also higher than that of *E. coli*. For instance, the D_{MTS} values of *L. monocytogenes* were approximately 1-fold higher than that of *E. coli* in both fruit juice medium at constant pressure and amplitude combination (200 kPa, 110 µm) at all temperatures. Moreover, at 100 kPa pressure under same amplitude conditions, similar differences (app.1-fold) were observed between both D_{MTS} values of selected strains at different treatment temperatures. Therefore, under similar processing conditions greater destruction of *E. coli* O157:H7 was found as compared to *L. monocytogenes* STCC 5672. Significant difference was found between the D_{MTS} values of both bacteria ($p \leq 0.05$, Appendix B).

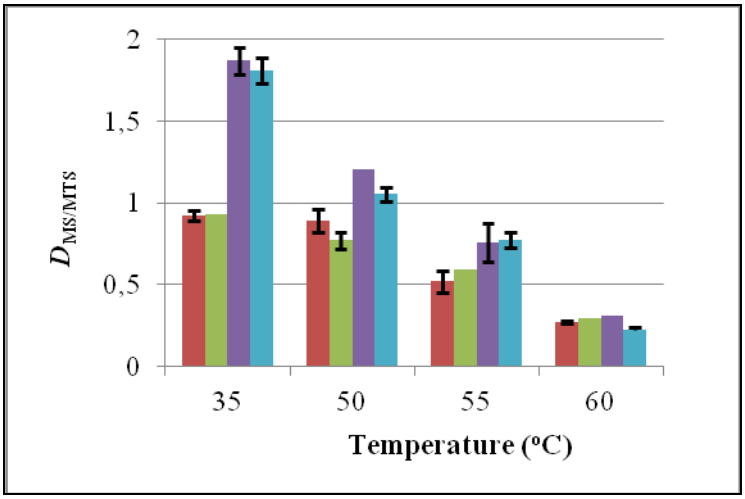

Figure 3.70. Effect of temperature on the resistances of *L.monocytogenes* STCC 5672 in apple (blue bar) and orange (purple bar) juices and *E.coli* O157:H7 in apple (red bar) and orange (green bar) juices by MTS (20 kHz, 110 µm) under constant pressure (200 kPa) at different temperatures [35, 50, 55 and 60 °C].

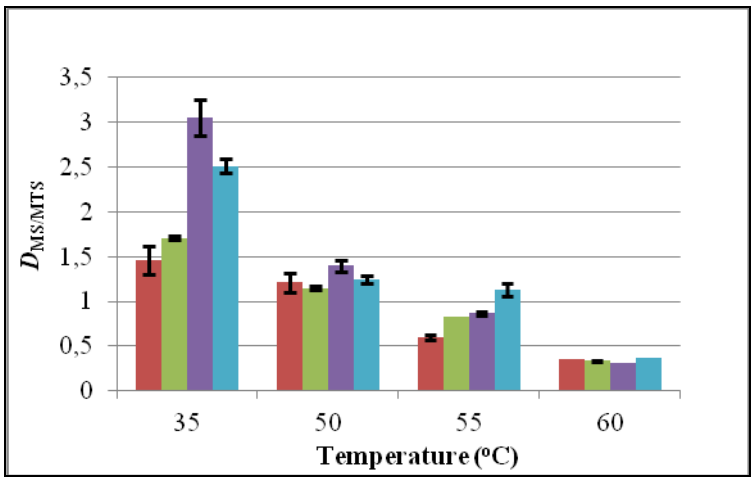

Figure 3.71. Effect of temperature on the resistances of *L. monocytogenes* STCC 5672 in apple (blue bar) and orange (purple bar) juices and *E.coli* O157:H7 in apple (red bar) and orange (green bar) juices by MTS (20 kHz, 110 µm) under constant pressure (100 kPa) at different temperatures [35, 50, 55 and 60 °C].

As mentioned previously, whereas the survival curves to MTS in acidic fruit juices were log-linear, the survival curves to TT in acidic fruit juices showed a shoulder. The description and quantification of these deviations from linearity - shoulders – required the application of a non-linear inactivation model (Weibull model with Mafart equation, Mafart et al., 2002) as described earlier in Materials and Methods section 2.3.3.4. At 50 and 55 ˚C, the shoulder profiles were observed in apple and orange juices (Figures 3.72, 3.73, 3.74, 3.75, 3.76, and 3.77). Figures 3.78, 3.79, 3.80, and 3.81 show the survival curves of the selected microorganisms to TT (60 ˚C), MS (200 kPa, 110 µm, 35 ˚C), and to the simultaneous application of both hurdles (MTS, 200 kPa, 110 µm, 60 ˚C) in both fruit juices. As pointed out above, only survival curves to TT for both strains showed shoulders. The main aim of using these figures to describe differences between the inactivation rates to TT, in which a concave downward profile was observed, and of MS and MTS, which were an exponential function of treatment time.

Furthermore, at 50 ˚C, the comparison of survival curves of MS/MTS and TT were not possible, because of the higher *D* values of *E. coli* in apple and orange juices. Therefore, the survival curve graphs of *E. coli* at 50 ˚C in both juices were not plotted like as other survival curves at 55 and 60 ˚C.

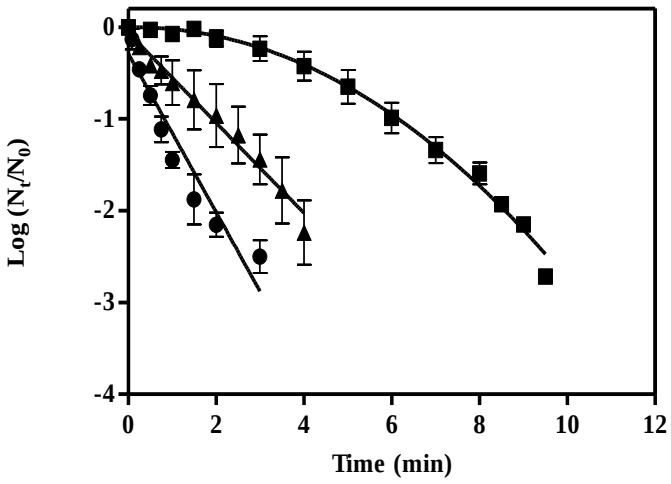

Figure 3.72. Survival curves of *L. monocytogenes* STCC 5672 subjected to thermal (50 ˚C, 200 kPa) (■), MS (35 ˚C, 200 kPa, 110 μm) (▲), and MTS (50 ˚C, 200 kPa, 110 μm) (●) treatments in apple juice.

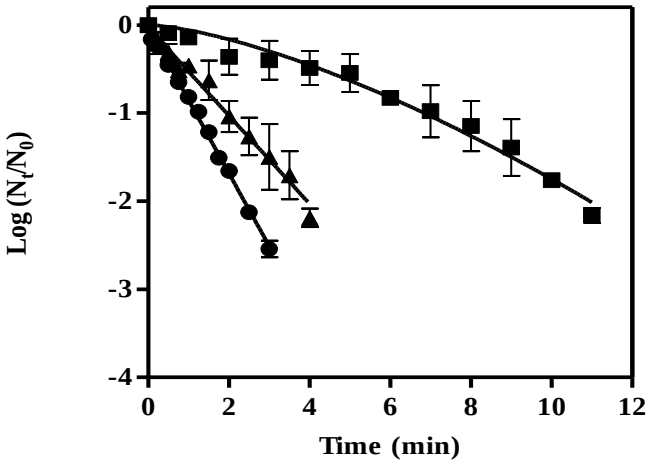

Figure 3.73. Survival curves of *L. monocytogenes* STCC 5672 subjected to heat (50 ˚C, 200kPa) (■), MS (35 ˚C, 200 kPa, 110 μm) (▲), and MTS (50 ˚C, 200 kPa, 110 μm) (●) treatments in orange juice.

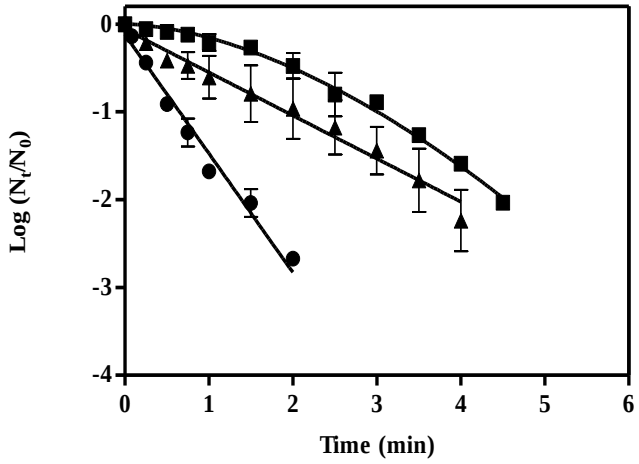

Figure 3.74. Survival curves of *L. monocytogenes* STCC 5672 subjected to thermal (55 ˚C, 200 kPa) (■), MS (35 ˚C, 200 kPa, 110 µm) (▲), and MTS (55 ˚C, 200 kPa, 110 µm) (●) treatments in apple juice.

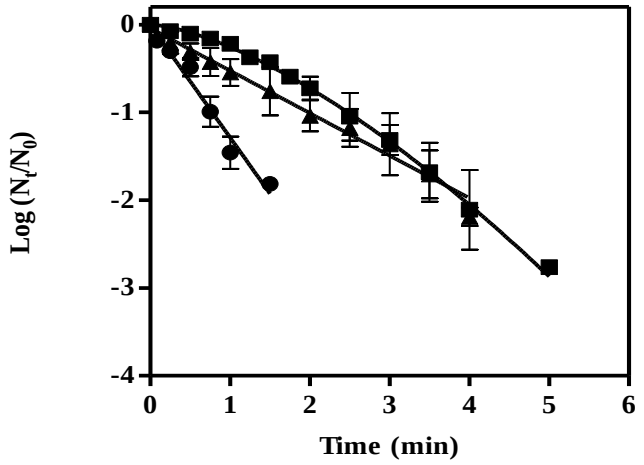

Figure 3.75. Survival curves of *L. monocytogenes* STCC 5672 subjected to heat (55 ˚C) (■), MS (35 ˚C, 200 kPa, 110 µm) (▲), and MTS (55 ˚C, 200 kPa, 110 µm) (●) treatments in orange juice.

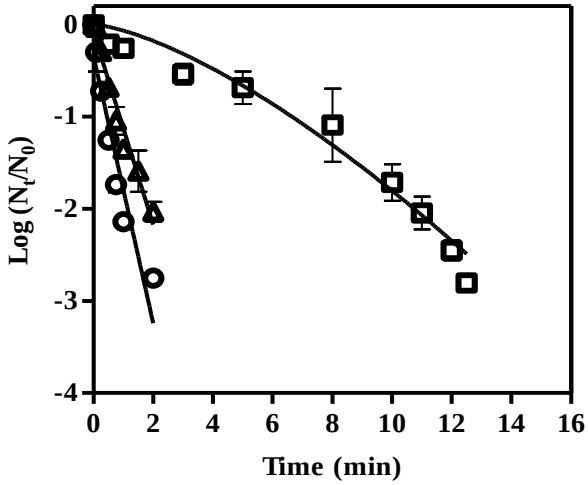

Figure 3.76. Survival curves of *E. coli* O157:H7 subjected to thermal (55 ˚C, 200 kPa) (□), MS (35 ˚C, 200 kPa, 110 µm) (Δ), and MTS (55 ˚C, 200 kPa, 110 µm) (○) treatments in apple juice.

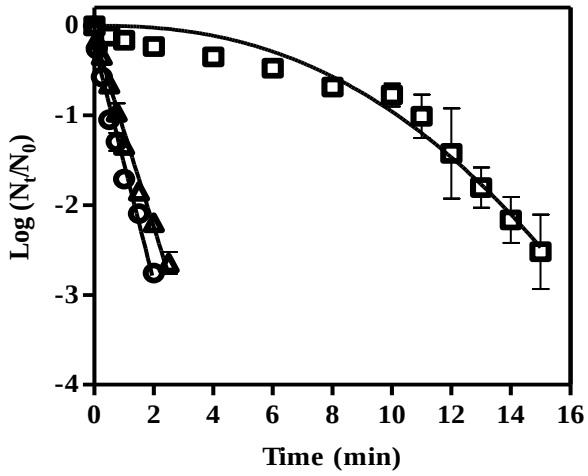

Figure 3.77. Survival curves of *E. coli* O157:H7 subjected to heat (55 ˚C, 200 kPa) (□), MS (35 ˚C, 200 kPa, 110 µm) (Δ), and MTS (55 ˚C, 200 kPa, 110 µm) (○) treatments in orange juice.

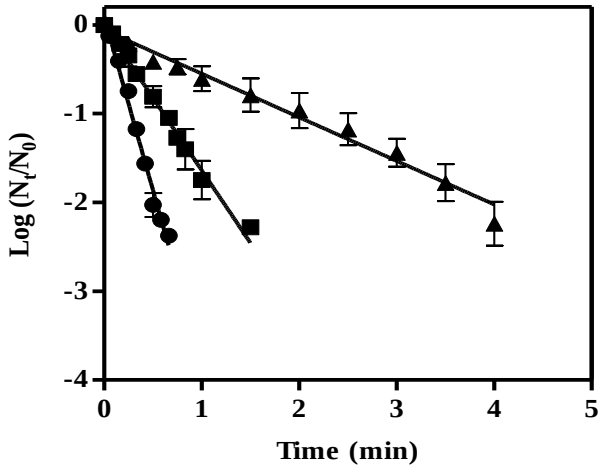

Figure 3.78. Survival curves of *L. monocytogenes* STCC 5672 subjected to thermal (60 ˚C, 200 kPa) (■), MS (35 ˚C, 200 kPa, 110 µm) (▲), and MTS (60 ˚C, 200 kPa, 110 µm) (●) treatments in apple juice.

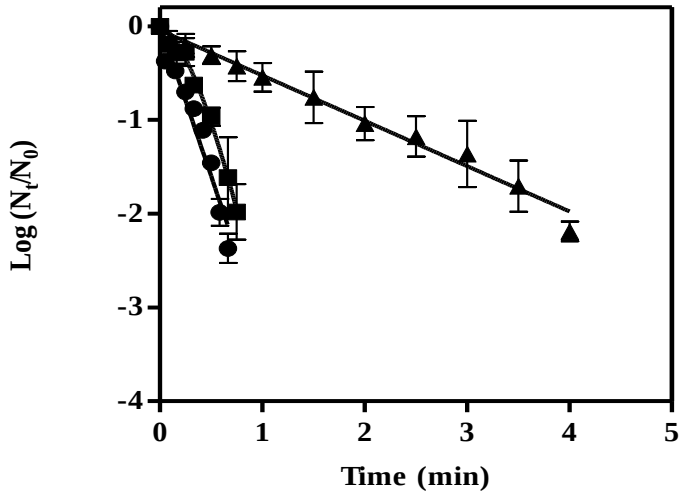

Figure 3.79. Survival curves of *L. monocytogenes* STCC 5672 subjected to thermal (60 ˚C, 200 kPa) (■), MS (35 ˚C, 200 kPa, 110 µm) (▲), and MTS (60 ˚C, 200 kPa, 110 µm) (●) treatments in orange juice.

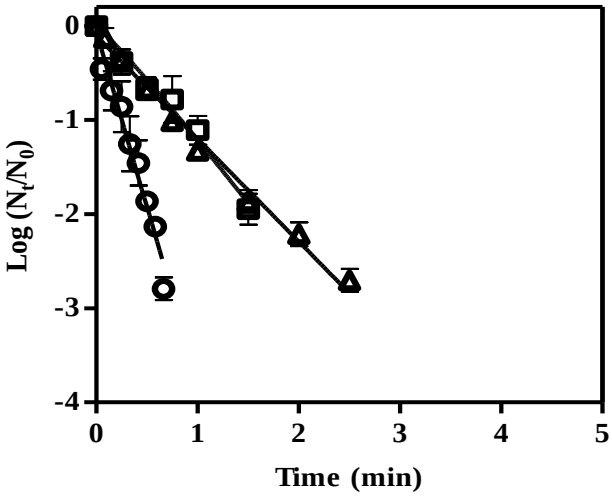

Figure 3.80. Survival curves of *E. coli* O157:H7 subjected to thermal (60 ˚C, 200 kPa) (□), MS (35 ˚C, 200 kPa, 110 µm) (Δ), and MTS (60 ˚C, 200 kPa, 110 µm) (○) treatments in apple juice.

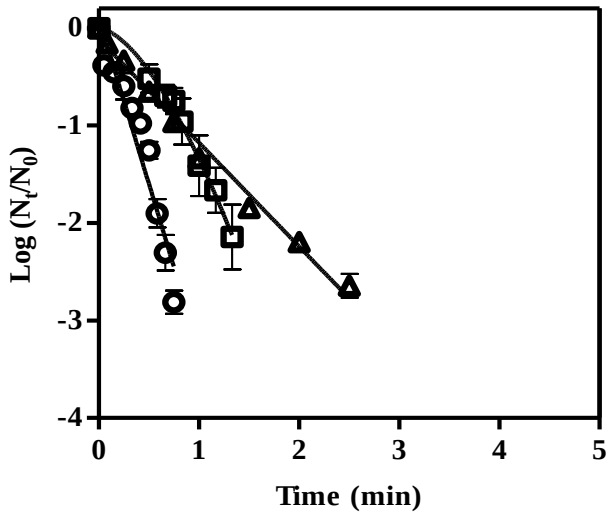

Figure 3.81. Survival curves of *E. coli* O157:H7 subjected to thermal (60 ˚C, 200 kPa) (□), MS (35 ˚C, 200 kPa, 110 µm) (Δ), and MTS (60 ˚C, 200 kPa, 110 µm) (○) treatments in orange juice.

The individual contributions of temperature and MS to the lethal effect of MTS treatments at different temperatures can be deduced from Figure 3.82 in apple juice and Figure 3.83 in orange juice for both strains. As seen in the figures, the lethal effect of MS for both microorganisms was influenced many environmental and process parameters, but not influenced by treatment temperature up to 50°C, the threshold value for both treatments medium. Therefore, D_{MS} values would be constant. However, above threshold temperature, when the treatment temperature increased, the lethality of MTS treatment increased. The experimental data given in Table 3.11 demonstrate that MS became MTS at 50°C in both treatment media for both selected strains. MS depends on cavitation intensity and heat has no lethal effect on this process. The inactivation by MS was independent of temperature up to a threshold temperature above which the rate of inactivation quickly increased with temperature. MTS is also a similar process including the combination lethal effect of ultrasound under pressure and heat. This limiting temperature distinguishes between MS and MTS treatments and it is dependent on the microorganism and the treatment media (Raso et al., 1998 a, 1998b; Pagán et al., 1999a; Álvarez et al., 2003, 2006; Arroyo et al., 2011a, 2011b; 2012). This threshold temperature for *L. monocytogenes* and *E. coli* was coincident with that reported for *Y. enterocolitica* (Raso et al., 1998b) and *L. monocytogenes* (Pagán et al., 1999b) in pH 7.0 citrate-phosphate buffer, but lower than that of *E. coli* (Lee et al., 2009b) in pH 7.0 phosphate buffer (54°C). Above 50°C, D_{MTS} values quickly decreased with temperature in both media. However, the rate of decrease of the *D* values with the temperature was faster for TT than for MTS in Figures 3.82 and 3.83. Thus, raising the treatment temperature of the combined treatment from 35 to 60 °C caused an 8-fold and a 6-fold decrease in the *D* values of *L. monocytogenes* in apple and orange juices, respectively, and a 3-fold decrease in the *D* values of *E. coli* in apple and orange juices.

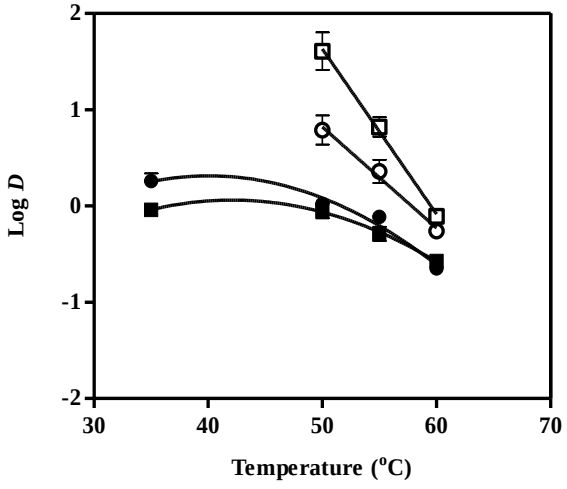

Figure 3.82. Effect of temperature on *L. monocytogenes* STCC 5672 inactivation by heat (○) and MS/MTS (●) and on *E. coli* O157:H7 inactivation by heat (□) and MS/MTS (■) treatments in apple juice.

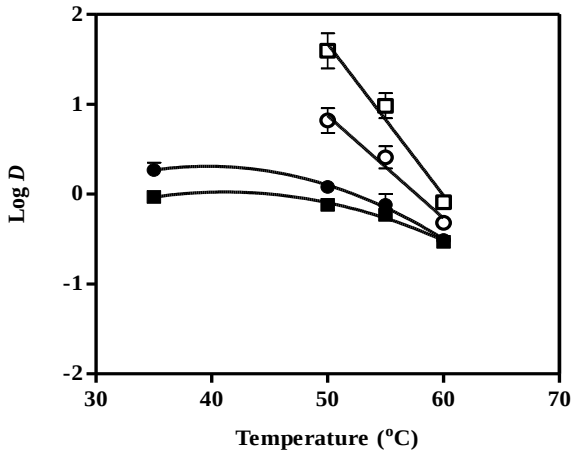

Figure 3.83. Effect of temperature on *L. monocytogenes* STCC 5672 inactivation by heat (○) and MS/MTS (●) and on *E. coli* O157:H7 inactivation by heat (□) and MS/MTS (■) treatments in orange juice.

Most of the data published indicates that the combination of heat and ultrasound under pressure would have an additive effect, i.e., that the lethal effect of MTS would be the result of adding the inactivation rates of TT and MS acting simultaneously but independently (Condón, et al., 2011). It has been described for *Y. enterocolitica* (Raso et al., 1998), *Salmonella* Enteritidis and *Aeromonas hydrophila* (Pagán et al., 1999b) in pH 7.0 buffer, and *L. monocytogenes* in apple cider (Baumann et al., 2005). According to this hypothesis, the occurrence of an additive effect has been attributed to the different mechanism of inactivation of both technologies (Raso et al., 1998a, 1998b; Condón et al., 2011). Although additive effect was described for combination of some individual preservation techniques, synergistic effect was selected as an alternative effect according to food quality and safety by some researchers (Raso & Barbosa - Cánovas, 2003). For synergistic effect, it was reported that the lethal effect of MTS was higher than expected, i.e., it was found under particular treatment conditions (Raso et al., 1998a; Pagán et al., 1999a). For conventional preservation treatments, optimal microbial control is achieved through the hurdle concept, with synergistic effects resulting from different components of the microbial cell being targeted simultaneously (Leistner and Gorris, 1995; Leistner, 2000).

The occurrence of additive or synergistic effects would depend on the microorganism investigated, the range of temperatures, and the treatment media tested (Arroyo et al., 2012). The relationship between the experimental and theoretical MTS values was also illustrated to establish the origin of the interactions between ultrasound under pressure and heat for the inactivation of *L. monocytogenes* and *E. coli* cells in acidic medium in Figures 3.84 and 3.85. In this study, it is obviously shown that the experimental and theoretical MTS data of both selected strains were not corresponded to each other as related to Figure 3.84 for apple juice and to Figure 3.85 for orange juice. Additionally, in

order to determine whether this increase in lethality by MTS process over TT was due to an additive or to a synergistic effect, the experimental $4D_{MTS}$ values were compared with their corresponding theoretical $4D_{MTS}$ values in Table 3.12. Therefore, the results demonstrated that heat and ultrasound acted synergistically to inactivate *L. monocytogenes* and *E. coli* cells by MTS in apple and orange juices at all treatment temperatures. The maximum synergism was observed when inactivating *L. monocytogenes* in apple juice by MTS at 60 ºC: the experimental $4D_{MTS}$ value was a 45 % lower than the theoretical $4D_{MTS}$ is corresponding to the sum of the effectiveness of heat and MS treatment acting simultaneously but independently. From published data, it can be reached a conclusion that some microorganisms studies, such as *Bacillus subtilis* (Raso et al., 1998b); heat-shocked *L. monocytogenes* (Pagán et al., 1999c); *Enterococcus faecium* (Pagán et al., 1999b); *C. sakazakii* (Arroyo et al., 2011b) in pH 7.0 buffer and milk, and in apple juice (Arroyo et al., 2012), would have a synergistic effect.

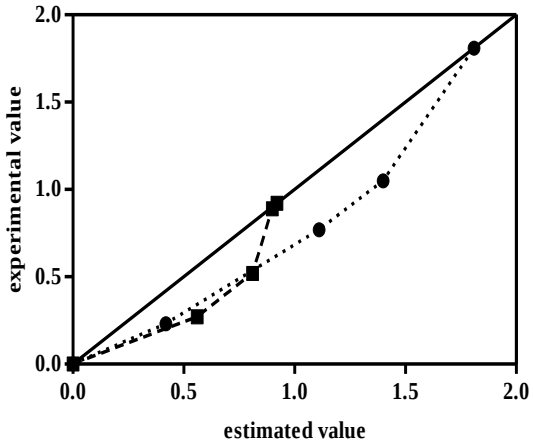

Figure 3.84. Comparison between the theoretical and the experimental D values determined for the inactivation of *L. monocytogenes* STCC 5672 (●) and *E. coli* O157:H7 (■) by MTS (200 kPa, 110 μm) treatments at different temperatures in apple juice. Theoretical data were calculated with the equation 9: Theoretical $4D_{MTS} = (4D_{TT} \times 4D_{MS}) / (4D_{TT} + 4D_{MS})$. The area between the continuous line (theoretical data) and the discontinuous line (experimental data) represents the magnitude of synergistic effect observed.

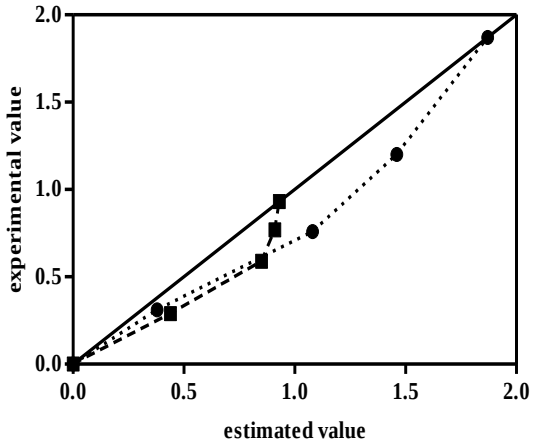

Figure 3.85. Comparison between the theoretical and the experimental D values determined for the inactivation of *L. monocytogenes* STCC 5672 (●) and *E. coli* O157:H7 (■) by MTS (200 kPa, 110 μm) treatments at different temperatures in orange juice. Theoretical data were calculated with the equation 9: Theoretical $4D_{MTS}= (4D_{TT} X 4D_{MS}) / (4D_{TT} + 4D_{MS})$. The area between the continuous line (theoretical data) and the discontinuous line (experimental data) represents the magnitude of synergistic effect observed.

On the other hand, it is noteworthy to point out that despite *L. monocytogenes* STCC 5672 should be considered as the target microorganism under MS treatments and the same for *E. coli* O157:H7 under heat treatments, no significant differences were found between the D_{MTS} values for both strains at 60 ºC in both juices ($p<0.05$). The lower treatment temperature to achieve a required level of inactivation together with this reduction in the interspecific differences in resistance is the two main advantages of using MTS treatments to process acidic fruit juices.

Table 3.12. Relationship between temperature and experimental and theoretical $4D_{MTS}$ values (calculated with Eq. 2.9) of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 in apple and orange juices and the synergistic effect of the combined MTS treatment at different temperatures (calculated with Eq. 2.10).

Treatment media	Microorganisms	Temperature (^{o}C)	$4D_{MS}$ (min)	$4D_{TT}$ (min)	Theoretical $4D_{MTS}$ (min)	Experimental $4D_{MTS}$ (min)	% synergism
Apple juice	*L. monocytogenes* STCC 5672	35	7.24	-	-	-	-
		50	-	24.64	5.60	4.20	24.94
		55	-	9.16	4.04	3.08	23.83
		60	-	2.20	1.69	0.92	45.47
	E. coli O157:H7	35	3.68	-	-	-	-
		50	-	162.48	3.60	3.56	1.07
		55	-	26.52	3.23	2.04	36.87
		60	-	3.12	1.69	1.08	36.04
Orange juice	*L. monocytogenes* STCC 5672	35	7.48	-	-	-	-
		50	-	26.64	5.84	4.80	17.81
		55	-	10.36	4.34	3.04	30.01
		60	-	1.92	1.53	1.24	18.84
	E. coli O157:H7	35	3.72	-	-	-	-
		50	-	157.52	3.63	3.08	15.25
		55	-	38.48	3.39	2.36	30.43
		60	-	3.24	1.73	1.16	33.01

3.3. Enzyme Inactivation

It has been known for nearly 80 years that US can be employed as a method of enzyme inactivation. Because of cavitation, Chambers (1937) firstly reported US inactivation of pure pepsin. The effects of ultrasonic waves on enzymes are very complex, and activation or inactivation can take place depending on the conditions and intensity of the treatment (McClements, 1995; Kadkhodaee, 2006), as well as on the nature of the enzyme (Sala et al., 1995; Kadkhodaee, 2006). Several authors (Ordóñez, et al., 1984; López et al., 1994, 1998; López and Burgos, 1995a, 1995b; Sala et al., 1995; Vercet et al., 1997, 1999, 2001; Ku et al., 2000; Mañas et al., 2000b; Villamiel and Jong, 2000b; Kuldiloke, 2002; Raviyan et al., 2005; Wu et al., 2008; Yaldagard et al., 2008; Tefere et al., 2009) have extensively reported the destructive effect of ultrasound on food enzymes (PME, PPO, PG, PE, LOX, lipase, protease, etc.) either individually or in combination with other inhibitory factors such as heat, pressure, pH, and sanitizers. The effect of ultrasound alone is very low, and long exposure times are required for enzyme inactivation (Thakur and Nelson, 1997; Kadkhodaee, 2006; O'Donnell et al., 2010). Therefore, the most recent investigations have studied the increase of effectiveness of US enzyme inactivation using heat and pressure (mano-thermo-sonication, MTS) (Sala et al., 1995).

3.3.1 Temperature Profile During Enzymes Inactivation by US

The ultrasound application responsible for the increase of temperature in processed medium because of cavitation phenomena (Śliwiński, 2001). An ultrasonic processor transforms the electrical energy into other kind of energies such as mechanical energy, acoustical energy, cavitation energy, and thermal energy. Löning et al. (2002) mentioned that almost 75% of acoustic energy transmitted by converter might be subsequently transformed into thermal energy of a system processed. Therefore, monitoring of US treatment thermal changes within treatment medium along with searching for temperature profiles are important for observation and evaluation of max temperature, which exposed to enzymes. Figures 3.86, 3.87, 3.88 and 3.89 present the temperature changes in US treated apple and orange juices. Before applying US at selected combinations, the initial temperatures of untreated samples were measured. The initial temperature of sonicated medium was 10°C. At 200 kPa, three different amplitudes (90 μm, 110 μm, and 130.5 μm) were studied in apple and orange juices (Figures 3.86 and 3.87). To observe the effect on pressure on the temperature profile, three different pressures at constant amplitude (130.5 μm) were applied in apple (Figure 3.88) and orange (Figure 3.89) juices. The higher temperatures were recorded for the highest ultrasound amplitude (130.5 μm) and the highest pressure (200 kPa), which were 48 °C for apple juice and 52 °C for orange juice, respectively. According to statistical analysis of US treated apple and orange juices, amplitude and pressure have significant effect ($p \leq 0.05$) on the final temperatures of acidic fruit juices. Kobus (2006) and Basmacı (2010) reported same temperature profiles of US treated carrots and honey, respectively. They mentioned that the highest temperatures were measured at the max level conditions such as the highest-level amplitude or US intensity and the highest probe diameter.

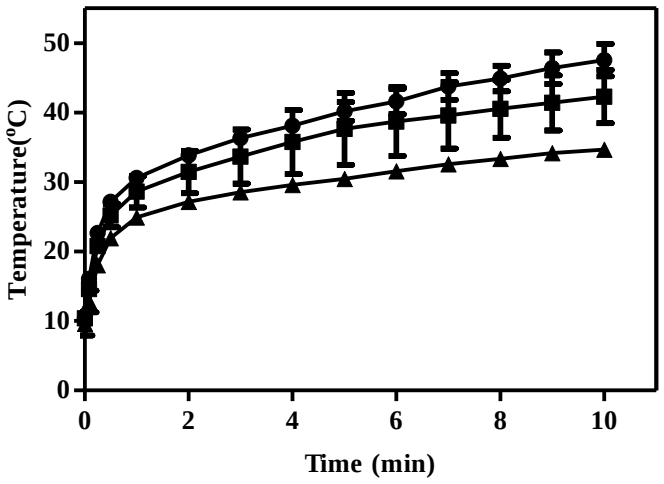

Figure 3.86. Temperature profile of US (200 kPa) treated apple juice at different amplitudes (90 µm (▲), 110 µm (■), and 130.5 µm (●))

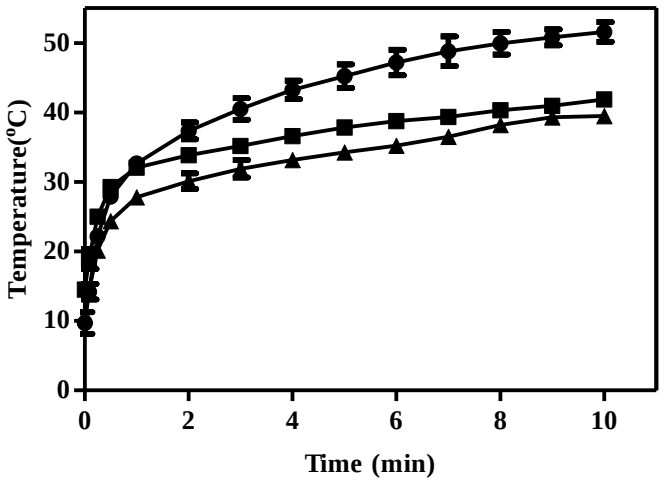

Figure 3.87. Temperature profile of US (200 kPa) treated orange juice at different amplitudes (90 µm (▲), 110 µm (■), and 130.5 µm (●))

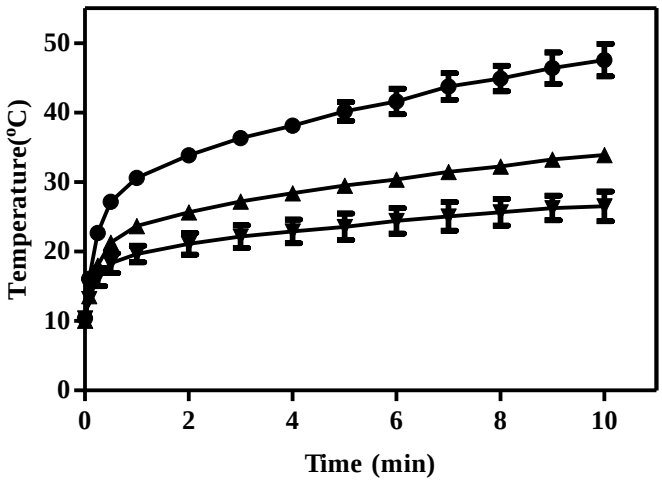

Figure 3.88. Temperature profile of US (130.5 μm) treated apple juice at different pressures (0 kPa (▼), 100 kPa (▲), and 200 kPa (●))

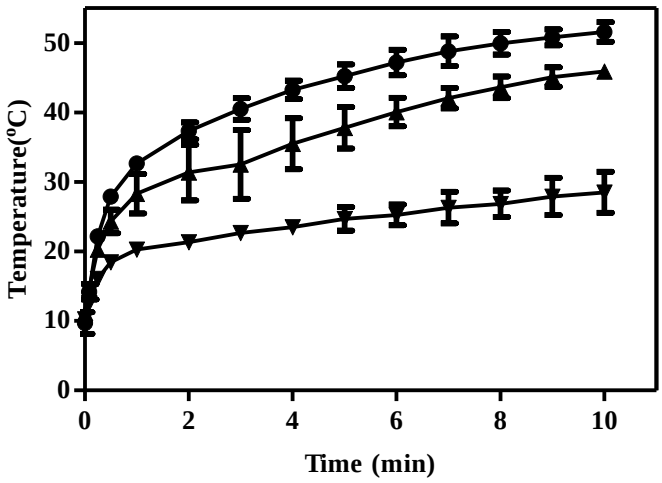

Figure 3.89. Temperature profile of US (130.5 μm) treated orange juice at different pressures (0 kPa (▼), 100 kPa (▲), and 200 kPa (●))

3.3.2 MS Experiments

MS is a process combining the ultrasound and pressure simultaneously to use for enzyme inactivation. Although there were some studies related to enzymes inactivation by US (Ordóñez, et al., 1984; López et al., 1994, 1998; López and Burgos, 1995a, 1995b; Sala et al., 1995; Thakur and Nelson, 1997; Vercet et al., 1997, 1999, 2001, 2002a, 2002b; De Gennaro et al., 1999; Ku et al., 2000; Villamiel and Jong, 2000b; Kuldiloke, 2002; Raviyan et al., 2005; Mañas et al., 2006; Yaldagard et al., 2008; Wu et al., 2008; Ganjloo et al., 2009; Kuldiloke and Eshtiaghi, 2009; Tefere et al., 2009; Tiwari et al., 2009; Ercan and Soysal, 2011; Fontales et al., 2012; Cheng et al., 2013), there were limited studies to investigate the inactivation of enzymes by ultrasound under pressure. Therefore, the effect of MS on inactivation of PPO in apple juice and PME in orange juice were investigated in this study.

3.3.2.1 Effect of Ultrasonic Waves Amplitudes on MS Inactivation of PPO and PME

The effect of amplitude of ultrasonic waves on the activities to MS of the selected enzymes, PPO and PME, in freshly squeezed apple and orange juices was studied. Different amplitude-pressure combinations with three different amplitudes (90, 110, and 130.5 μm) under two different pressures (100, and 200 kPa) were applied in both juices. As can be seen in Figures 3.90, 3.91 (for PPO), and 3.92 (for PME), a decrease in the residual activity of PPO in apple juice and of PME in orange juice were caused by increasing the amplitude.

Figures 3.90 and 3.91 presented the residual activity change of PPO enzyme in freshly squeezed apple juice at 100 and 200 kPa pressures, respectively. Control treatment was performed at room temperature (25 °C) and ambient pressure. In the control treatment, the activity of PPO still closed to initial activity at the end of the treatment time (10 min). On the other hand, when MS (200 kPa) was applied for 10 min treatment time at ultrasonic wave amplitudes of 46.5, 68.25, 90 and 110 μm, the inactivation of PPO were 43, 60, 88.5 and 90.1 %, respectively. Moreover, 98.7 % PPO inactivation was observed at 130.5 μm amplitude for 200 kPa. At low amplitudes of US where PPO was partially inactivated. Therefore, the 46.5 μm of amplitude has less effective on the inactivation of PPO than other amplitudes. It can be seen from the inactivation plot (Figure 3.91), there was sharp decrease in enzyme activity for the first 1 min at 90, 110, and 130.5 μm amplitudes. After 1 min of MS treatment, the decreasing of activity was smaller but effective. In the Figure 3.90 (100 kPa pressure), the effect of amplitude is seen more clearly. When MS was applied for 10 min, 47, 75 and 90.4% PPO inactivation were observed at US wave amplitudes of 90, 110, and 130.5 μm, respectively. The enzyme activity decreased with increasing the magnitude of amplitude and D_{MS} values of PPO also decreased as given in Table 3.13. It can be easily seen from the inactivation plots that the increase in the amplitude of ultrasonic waves had a significant effect ($p \leq 0.05$) on the inactivation of PPO in freshly squeezed apple juice under constant pressure (Figures 3.90 and 3.91). In addition to that the D_{MS} values of PPO of over sonication amplitude range (46.5 - 130.5 μm) used in the study were significantly different at any of the pressures studied ($p \leq 0.05$) (Anova table in Appendix C). Ercan and Soysal (2011) studied the

effect of US on tomato POD activity at five different US amplitudes (3, 5, 8, 10, and 15 μm) for 20-150 seconds. The authors reported that as ultrasonic wave amplitude increased, the inactivation rate increased. Our results are also in agreement with the results of López et al. (1994); López and Burgos (1995a, 1995b);Thakur and Nelson (1997); Vercet et al. (2002a, 2002b); Raviyan et al. (2005); Yaldagard et al. (2008); Tiwari et al. (2009).

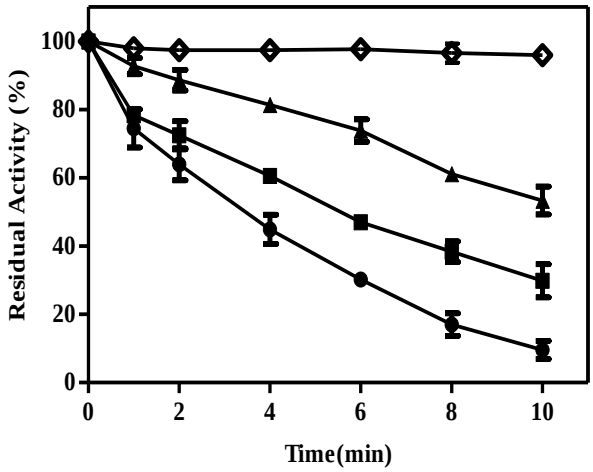

Figure 3.90. Residual activity of PPO in apple juice by ultrasound under pressure (MS treatment; 35 °C, 100 kPa) for three different amplitudes [90 μm (▲), 110 μm (■) and 130.5 μm (●)] and by control treatment [25 °C, ambient pressure (◊)].

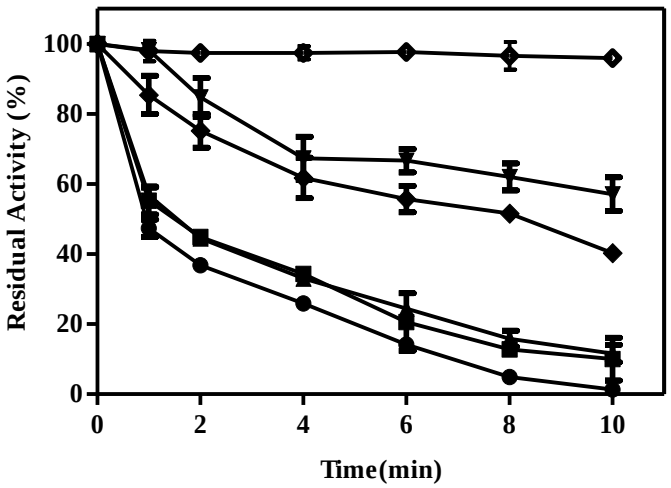

Figure 3.91. Residual activity of PPO in apple juice by ultrasound under pressure (MS treatment; 35 °C, 200 kPa) for five different amplitudes [46.5 µm (▼), 68.25µm (♦), 90 µm (▲), 110 µm (■) and 130.5 µm (●)] and by control treatment [25 °C, ambient pressure (◊)].

In order to in investigate the effect of ultrasonic wave amplitudes, freshly squeezed apple juice were sonicated at the different amplitudes. Log residual activity of PPO *vs.* time (min) graphs of related to amplitude plotted in Figure 3.92 for 100 kPa and Figure 3.93 for 200 kPa. From the figures, the enzyme residual activity decreased with increasing the US wave amplitude. Typically, the log residual activity decreased from -0,244 for 90 µm to -1,906 for max amplitude of 130.5 µm at 200 kPa at the end of the 10 min processing time. López et al. (1994) and Cheng et al. (2013) reported similar results for mushroom PPO in a model solution. In all the experiments, the PPO inactivation due to MS treatment follows first order kinetics according to data reported into the literature (López et al, 1994, 1998; López and Burgos, 1995a, 1995b; De Gennaro et al., 1999; Vercet et al., 1999, 2001; Raviyan et al., 2005; Wu et al., 2008; Yaldagard et al., 2008; Terefe et al., 2009; Ercan and Soysal, 2011; Cheng et al., 2013).

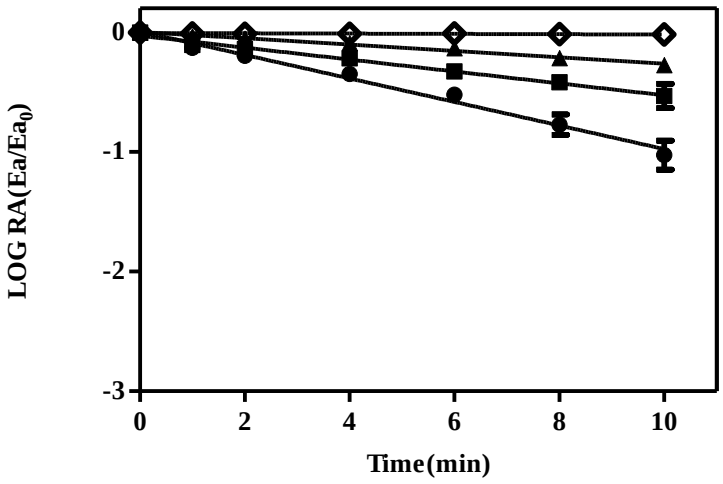

Figure 3.92. Inactivation rate of PPO in apple juice by MS (35 °C, 100 kPa) for three different amplitudes [90 µm (▲), 110 µm (■) and 130.5 µm (●)] and by control treatment [25 °C, ambient pressure (◊)].

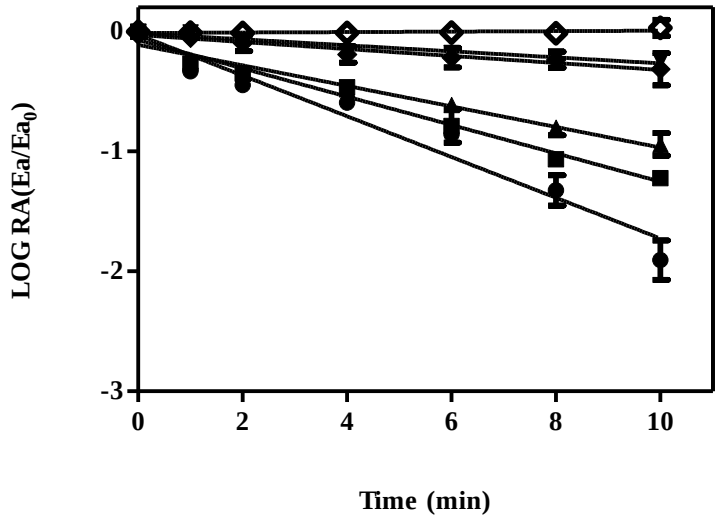

Figure 3.93. Inactivation rate of PPO in apple juice by MS (35 °C, 200 kPa) for five different amplitudes [46.5 µm (▼), 68.25 µm (♦), 90 µm (▲), 110 µm (■) and 130.5 µm (●)] and by control treatment [25 °C, ambient pressure (◊)].

Figure 3.94 shows the residual activity of PME enzyme in freshly squeezed orange juice. Three different amplitudes were used at constant pressure (200 kPa) and temperature (35 °C) in the MS experiments. Similar to PPO enzyme, control treatment was also performed at room temperature (25°C) and ambient pressure. At the end of the control treatment time (10 min), the activity of PME was still closed to initial activity value (98 %). When MS was applied at 200 kPa for 10 min, 60.77 %, 85.17 %, and 95.69 % PME inactivation were observed at US wave amplitudes of 90, 110, and 130.5 μm, respectively. Under constant pressure at 200 kPa, the activity of PME decreased with the increased in ultrasonic wave amplitudes and D_{MS} values of PME also decreased as given in Table 3.13. According to the inactivation plot of PME (Fig. 3.94), it can be seen that the increase in the amplitude of ultrasonic waves had a significant effect ($p \leq 0.05$) on the inactivation of PME in freshly squeezed orange juice. When comparing the inactivation rate of these conditions, it is obvious that higher amplitude value (130.5 μm) has greater inactivation effect on PME and D_{MS} value of its smaller than other amplitudes as given in the Table 3.13.

Whereas Wu et al. (2008) found that the *D* values of tomato PME over sonication amplitude range used (25-75 μm) were not significantly different at any of the temperatures studied (60, 65, and 70 °C), it has been reported that the amplitude significantly affected the effectiveness of the enzyme inactivation by TS or MTS in several studies (López et al., 1994; López and Burgos et al., 1995a, 1995b; Vercet et al., 2002b; Raviyan et al.,2005; Yaldagard et al., 2008; Tiwari et al., 2009). According to investigation of Vercet et al. (2002b), at constant pressure and temperature, the MTS inactivation of lipase and protease enzymes seems to be more amplitude dependent at amplitudes above 90 and 120 μm, respectively.

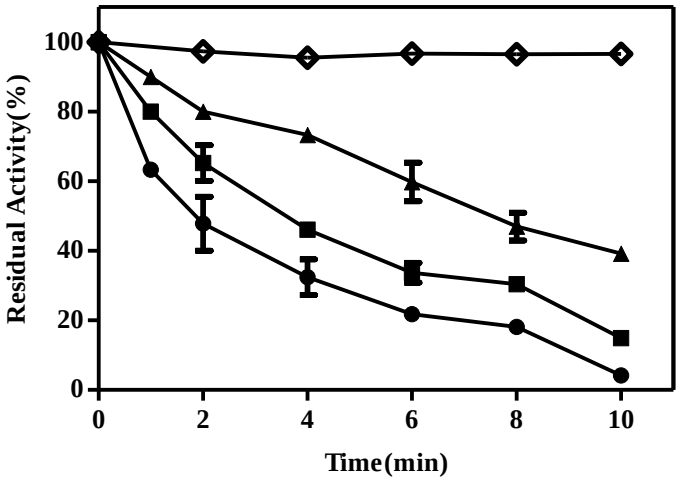

Figure 3.94. Residual activity of PME in orange juice by ultrasound under pressure (MS treatment; 35 °C, 200 kPa) for three different amplitudes [90 μm(▲), 110 μm(■) and 130.5 μm(●)] and control treatment [25 °C, ambient pressure (◊)].

Similar to MS inactivation of PPO, the plot of log residual activity of PME *vs*. ultrasonic time (min) fitted to linear regression model in Figure 3.95. The ultrasonic wave amplitudes increased, the inactivation rate of PME increased. This result is in agreement with the results of Ercan and Soysal (2011) for tomato peroxidase in a model solution by TS and Terefe et al. (2009) for PME in tomato juice by TS and López et al. (1998) for tomato PME by MTS.

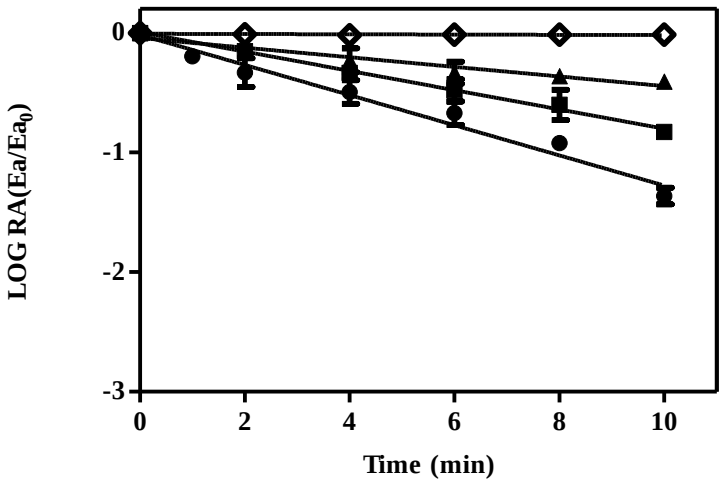

Figure 3.95. Inactivation rate of PME in orange juice by MS treatment (35 °C, 200 kPa) for three different amplitudes [90 μm (▲), 110 μm (■) and 130.5 μm (●)] and control treatment [25 °C, ambient pressure (◊)].

The effect of ultrasonic wave amplitude on the MS inactivation of selected enzymes was plotted in Figures 3.96. As can be observed, the D_{MS} values of both enzymes investigated decreased exponentially with the amplitude in the range between 90 and 130.5 μm at 200 kPa in apple and orange juices. The enzyme resistance of MS treatment was also affected by amplitude in acidic fruit juices. Similar to microbial MS inactivation in laboratory media, the linearity was found between the logarithm of the D_{MS} values and the ultrasonic wave amplitudes ($R^2 \geq 0.99$). Moreover, the magnitude of the effect of ultrasonic wave amplitude on the enzyme resistance was similar for PPO and PME in both juices. Although Kashkooli et al. (1980) have proved that enzyme inactivation by US at room temperature was related to acoustic micro streaming, Suslick (1988) reported that increased intensity of ultrasound irradiation increases the sonochemical reaction rates and therefore related to cavitational collapse. The validity of these phenomena is generally dependent on US amplitude, since the higher the US irradiation intensity the higher the temperature during collapse and consequently the sonochemical reaction rate (Mason and Lorimer, 1988). During the ultrasonic treatment at constant temperature and pressure, while increasing or decreasing of sonication amplitude affected the inactivation rate of enzymes and *D* values of treatments (López et al., 1994; López and Burgos, 1995a; Vercet et al., 2002b; Raviyan et al., 2005; Yaldagard et al., 2008; Wu et al., 2008). López

et al. (1994) reported that the greater amplitude, the higher the efficiency. This rule is obeyed by the two enzymes studied, and plot of Log (*D* values) *vs.* irradiation amplitude demonstrates (Figures 3.96 and 3.97) that log (*D* value) decreases linearly with increasing amplitude in all cases.

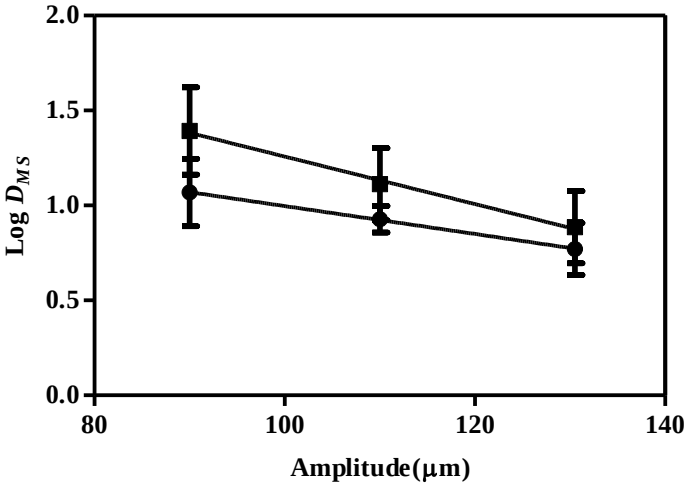

Figure 3.96. Effect of ultrasonic wave amplitudes on the inactivation rate of PPO (●) and PME(■) by ultrasound under pressure (MS treatment; 35 °C, 200 kPa) for three different amplitudes [90, 110 and 130.5 μm].

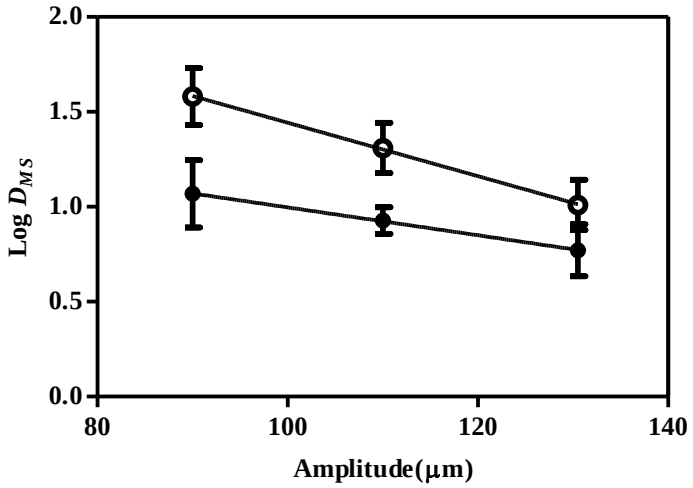

Figure 3.97. Effect of ultrasonic wave amplitudes on the inactivation rate of PPO by MS treatment (35 °C) at 200 kPa(●) and 100 kPa (o) for three different amplitudes [90, 110 and 130.5 μm].

Some authors (Raso et al., 1998a; Pagán et al., 1999a, 1999b, 1999c; Arroyo et al., 2011a; Condón et al., 2011) described amplitude linear equations to explain the relationship between the D_{MS} values of different microorganisms and the ultrasonic wave amplitudes in each treatment medium. These equations can also be described for PPO enzyme experiments in this investigation (Table 3.14). Although Log D_{MS} values of MS treatment had significant differences for both enzymes at all ultrasonic wave amplitudes ($p \leq 0.05$; App. C), no significant differences ($p > 0.05$) were found among the slopes of the regression lines for PPO enzyme (App C). Figure 3.97 is plotted to observe the effect of US amplitude on MS inactivation of PPO at different pressures (100 and 200 kPa). Three different ultrasonic wave amplitudes (90, 110 and 130.5 μm) were applied on PPO in freshly squeezed apple juice. From the Figure 3.97, the effect of amplitude on the inactivation of PPO is similar for both pressure cases at the same amplitude range. An exponential relationship between the logarithm of D_{MS} and amplitude was observed with PPO at different pressures. Moreover, there were significant differences between the magnitudes of ultrasound amplitude on the MS inactivation ($p \leq 0.05$) for PPO in apple juice, however, no significant differences ($p > 0.05$) were found among the slopes of the regression lines for the both pressures (App. C).

When comparing the effect of amplitude on inactivation rate of both enzymes in Figure 3.98, it is observed that US amplitudes have positive effect on the inactivation of PPO and PME enzymes and PME is relatively more resistance than PPO by MS. When increasing amplitude under constant pressure, the residual activities of both enzymes decreased. According to these results, the rate of decreasing residual activity would depend on the ultrasonic sensitivity of enzymes (Vercet et al., 2001; Villamiel and Jong, 2000b). Villamiel and Jong (2000b) mentioned that three different enzymes of milk (alkaline phosphatase, γ-glutamyltranspeptidase, and lactoperoxidase) presented different

sensitivity under ultrasonic inactivation treatments (20 kHz, 120 µm). Same authors also indicated ultrasound might increase the susceptibility of the active center of these enzymes to US or US - heat combine inactivation (TS). Vercet et al. (2001) reported that MTS sensitivity varied strongly for the different enzymes such as α-chymotrypsin and porcine lipase were more sensitive than phospholipase A_2 by MTS inactivation.

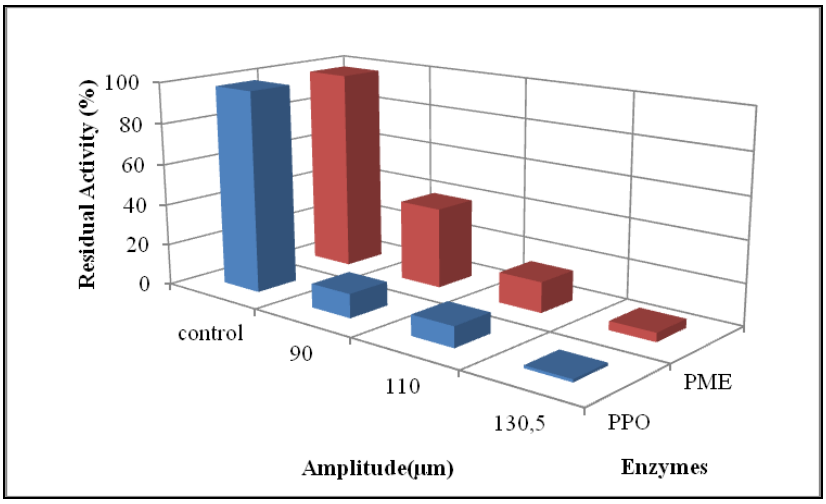

Figure 3.98. Effect of ultrasonic wave amplitudes on the residual activities of PPO (blue bar) and PME (red bar) by MS treatment (35 °C, 200 kPa) for three different amplitudes [90, 110, and 130.5 µm].

Table 3.13. Resistance parameters (D_{MS}) of PPO in apple juice and PME in orange juice to ultrasound treatments. ANOVA test ($p \leq 0.05$) was conducted within each group, as shown in Significant level column. To identify intergroup differences after significant differences in the ANOVA test, multiple comparisons were performed using Tukey's test. Values with the same superscript did not show statistically significant differences ($p > 0.05$).

Enzymes	Treatment Media	Temperature (°C)	Pressure (kPa)	Ampitude (μm)	Fit Parameters			
					D_{MS} Mean (min)	Standard Deviation (±)	R^2	Significant Level
PPO	Apple Juice	35	0	130.5	22.96	2.32	0.92	-
		35	100	90	37.89	3.13	0.95	$p \leq 0.05$
				110	20.22	2.14	0.96	
				130.5	10.24	1.08	0.97	
		35	200	46.5	40.03	3.72	0.87	$p \leq 0.05$
				68.25	35.47	7.88	0.74	
				90	11.71	0.78	0.95	
				110	8.46	0.07	0.97	
				130.5	5.90	0.37	0.95	
PME	Orange Juice	35	0	130.5	35.26	0.35	0.95	-
		35	100	130.5	22.18	2.25	0.91	-
		35	200	90	24.72	0.23	0.86	$p \leq 0.05$
				110	12.98	0.90	0.99	
				130.5	7.69	0.19	0.98	

Table 3.14. Best fit equations calculated for describing the effect of amplitude of ultrasonic waves on the MS inactivation of PPO in treated apple juice and PME in treated orange juice.

Enzymes	Treatment Media	Pressure (kPa)	Amplitude Linear Equation	R^2
PPO	Apple Juice	100	Log D_{MS} = - 0.01408 A + 2.851	0.99
		200	Log D_{MS} = - 0.00735 A + 1.733	0.99
PME	Orange Juice	200	Log D_{MS} = - 0.01252 A + 2.510	0.99

3.3.2.2 Effect of Pressure on MS Inactivation of PPO and PME

Effect of pressure on residual activities of PPO and PME at constant 130.5 µm amplitude and 35°C temperature in freshly squeezed apple and orange juices were given in the Figures 3.99 and 3.100, respectively. At constant amplitude (130.5 µm), the activity of PPO decreased as pressure increased. The inactivation of PPO was 65.49 %, 90.41 % and 98.69 % for 0, 100, and 200 kPa, respectively. The increase in pressure, D_{MS} values of PPO also decreased and were given in the Table 3.13. There were found significant differences ($p \leq 0.05$) between the D values of MS inactivation of PPO at constant amplitude (130.5 µm) (Table 3.13 and Appendix C). At 0 kPa, D_{MS} value of PPO (D_{MS}= 22.96 min) was approximately 2-fold and 4-fold higher than D_{MS} values of 100 kPa (D_{MS}= 10.24 min) and 200 kPa (D_{MS}= 5.90 min), respectively. 200 kPa pressure was more effective than other pressures on the inactivation rate of PPO.

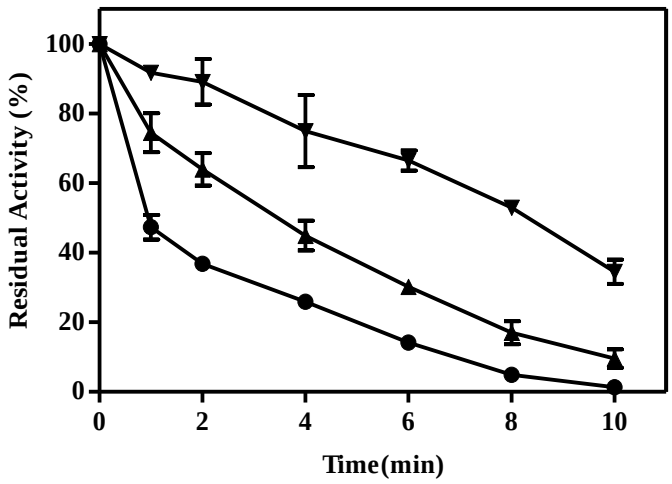

Figure 3. 99. Residual activity of PPO in apple juice by ultrasonication (35 °C, 130.5 µm) under the three different pressures [0 kPa (▼), 100 kPa (▲), and 200 kPa (●)].

In Figure 3.100, as a result, pressure increased at constant temperature (35 °C) and amplitude (130.5 µm), the activity of PME declined. From the Figures 3.101 to 3.104 can be seen that the greater the pressure, the higher efficiency of inactivation. The inactivation of PME was 46.77 %, 60.01 % and 95.69 % for 0, 100, and 200 kPa, respectively. Moreover, the D_{MS} values of PME declined as pressure increased. It was found significant difference ($p \leq 0.05$) between the D_{MS} value of PME in the selected condition. The D_{MS} value of PME at 0 kPa (D_{MS}= 35.26 min) was 1.5-fold and 4.5-fold higher than D_{MS} values of PME at 100 kPa (D_{MS}= 22.18 min) and 200 kPa (D_{MS}= 7.69 min), respectively. The effect of 200 kPa is greater than the effect of other pressures.

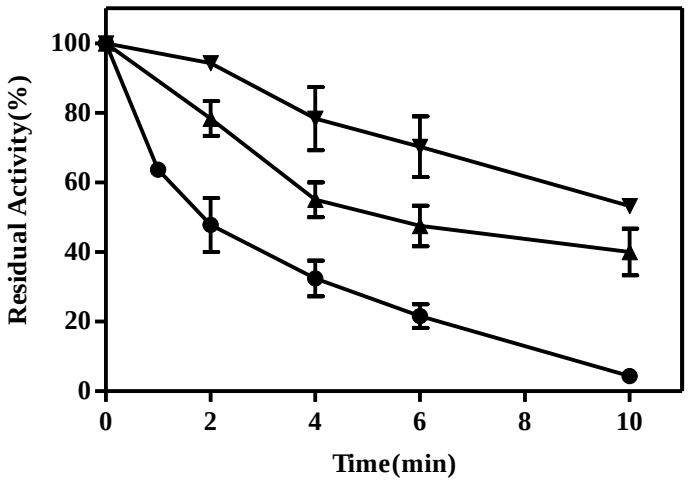

Figure 3.100. Residual activity of PME in orange juice by ultrasonication (35°C, 130.5 μm) under the three different pressures [0 kPa (▼), 100 kPa (▲) and 200 kPa (●)].

Manosonic inactivation of PPO in apple juice and PME in orange juice at constant amplitude (130.5 μm) under three different pressures (0, 100, and 200 kPa) showed apparent first order kinetics. The plots of the log residual activity *vs*. MS time fitted to linear models with high correlation coefficients (0.90 to 0.99) in Figure 3.101 for PPO and in Figure 3.102 for PME. The inactivations of PPO and PME have been attributed to an increase the pressure in treatment medium. Other authors found similar observations for TS inactivation (De Gennaro et al., 1999; Raviyan et al., 2005; Wu et al., 2008) and MTS inactivation (López et al., 1994; López and Burgos, 1995b; Vercet et al., 2001). Mañas et al. (2006) mentioned that MTS (117 μm, 20 kHz, 80°C) caused the inactivation of the egg white lysozyme in phosphate buffer, in a different degree depending on the pressure.

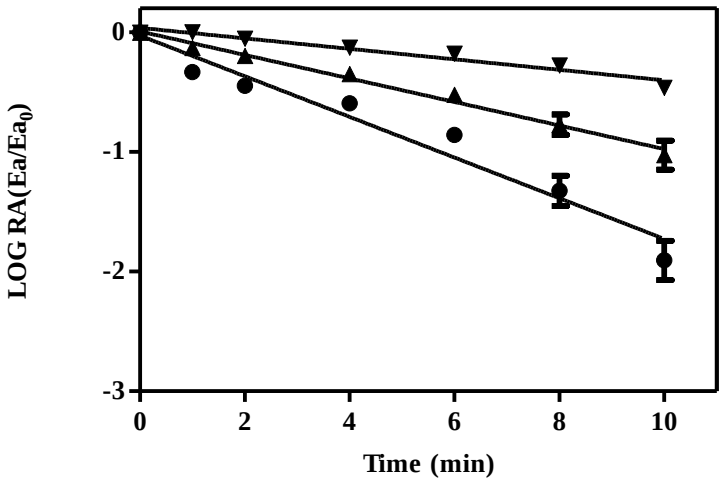

Figure 3.101. Inactivation rate of PPO in apple juice by US (35 °C, 130 μm) under the three different pressure [0 kPa (▼), 100 kPa (▲) and 200 kPa (●)].

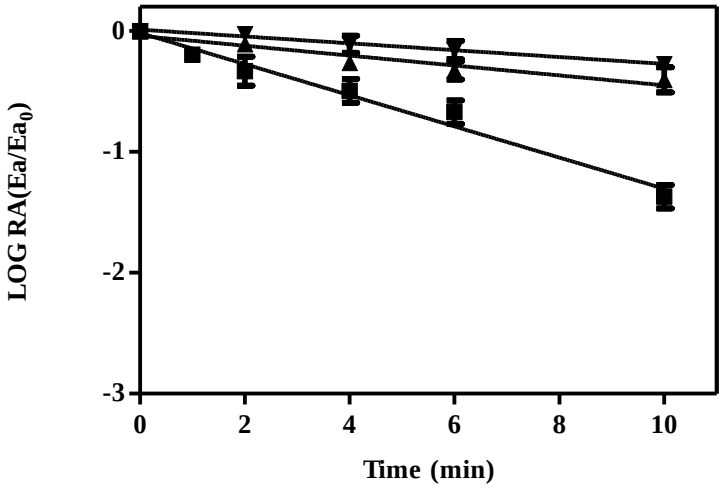

Figure 3.102. Inactivation rate of PME in orange juice by US (35 °C, 130 μm) under the three different pressure [0 kPa (▼), 100 kPa (▲) and 200 kPa (●)].

In literature, there was a lack of studies about inactivation effect of MS on enzyme. Therefore, the effect of pressure in the MS is still unclear. In literature, some authors mentioned that pressure effect on inactivation of enzymes was related to condition temperature. For instance, López et al. (1994) reported that increase in pressure in the range of 1.5-7 kPa increases the inactivation rate of LOX in the MTS treatment, however, *D* values remained unchanged at 70 °C constant temperature under 1.5-5 kPa pressure ranges, but dropped at 6-7 kPa pressure. Moreover, Kuldiloke (2002) mentioned that the differences between the *D* values of PE were higher, increase in the pressure from 100 to 300 kPa at low temperature (around 40°C), in contrary, the *D* values of PE remained stabil in the same pressure condition at 70°C constant temperature. According to these reports, pressure was not effective on the inactivation rate of enzymes at higher temperatures. Our results revealed that the increasing pressure caused greater inactivation rate of PPO and PME at sub lethal temperatures (35°C). In other words, the decreasing activity of enzymes by MS treatment was more pressure dependent at sub lethal temperatures. Suslick (1988) explained that during treatment, pressure increases the cavitation intensity of ultrasound. Because of increment in pressure, micro bubbles implosion increased and finally inactivation rate of enzymes increased.

The relationships between pressure during MS treatments (35˚C) at constant amplitude (130.5 µm) and Log D_{MS} values of PPO and PME are given in the Figure 3.103. Although similar graphs were generally observed in laboratory media for microorganisms such as *C. sakazakii* (Arroyo et al., 2011a), *L. monocytogenes* (Pagán et al., 1999a), and *Y. enterocolitica* (Raso et al., 1998b), López et al. (1994) and López and Burgos (1995a) presented this figure for LOX. This figure allows the adjustment or prediction of Log D_{MS} values of both enzymes at sub lethal temperature at different pressures like as microorganisms experiments (Raso et al., 1998a, 1998b; Pagán et al., 1999a, 1999b; Mañas et al., 2000a; Condón et al., 2011). According to Figure 3.103, the MS inactivation of PPO and PME increased exponentially by raising the relative pressure in apple and orange juices, respectively. In all cases and both fruit juices, the rise in inactivation of enzymes became progressively smaller as the pressure increased. As the pressure was increased from 0 to 100 kPa, the Log D_{MS} values of PPO and PME dropped to 26 % and 13 % of their original values, respectively. In the same way, when pressure was increased from 100 to 200 kPa, Log D_{MS} values decreased an extra 24 % and 35 %. Furthermore, under the same amplitude condition, the static pressure increased from 0 to 200 kPa, their Log D_{MS} values declined 57 % of their original values for both enzymes. Moreover, the magnitude of the effect of pressure was the same for both enzymes investigated in both fruit juices.

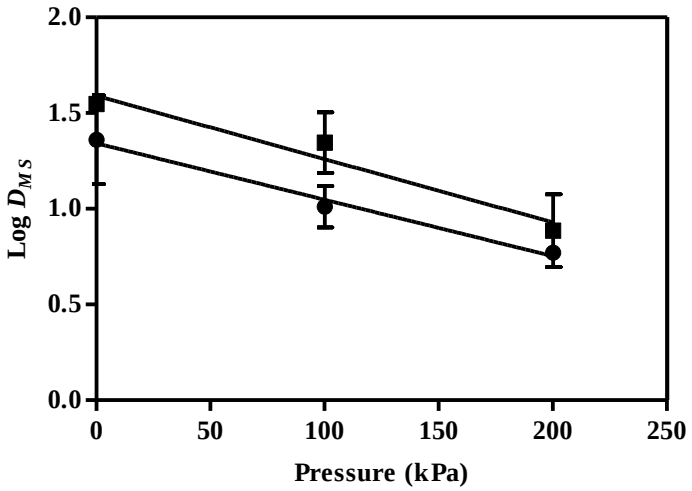

Figure 3. 103. Effect of pressure on the resistances of PPO (●) and PME (■) by MS treatment (35 ˚C) at constant amplitude (130.5 μm).

Residual activity (%) *vs.* pressure-amplitude combinations graph plotted in the Figure 3.104. This graph allows the comparison of resistance of both enzymes studied. From the figure, it is obviously seen that PME was relatively more resistant than PPO against the changing pressure in the MS treatment (130.5 μm, 35°C). In previously mentioned that the different structures of enzymes played important role in the ultrasonic sensitivity of enzymes (Villamiel and Jong, 2000b; Vercet et al., 2001). Sala et al. (1995) also mentioned that the effectiveness of ultrasound for enzyme inactivation depends on not only conditions, equipment, and the nature of the sonicating medium used but also on the type of enzyme.

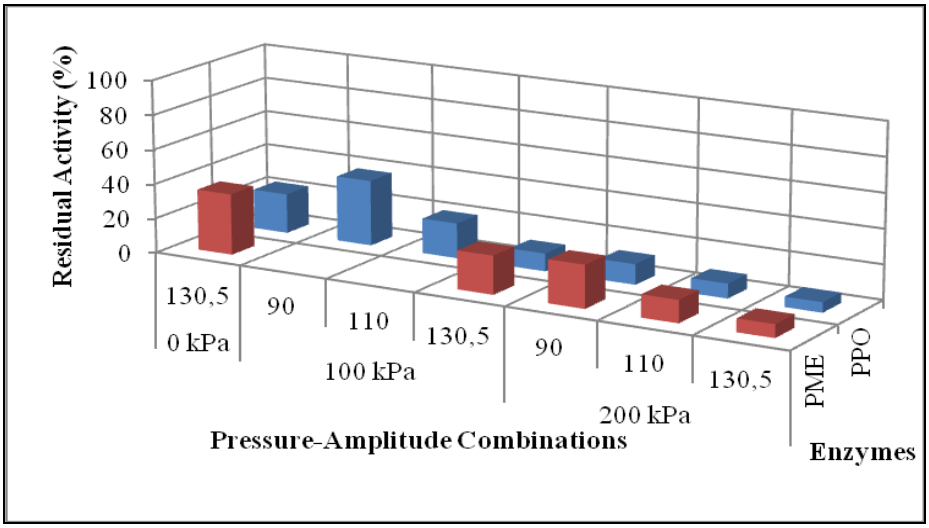

Figure 3.104. Effect of pressure on the residual activities of PPO (blue bar) and PME (red bar) by MS treatment (35 °C, 130.5 µm) under the three different pressures [0 kPa, 100 kPa, and 200 kPa].

To explain the relationship between MS inactivation of enzymes and pressure, a linear relationship was obtained as seen in the Table 3.15. The calculation of this equation allows the prediction of D_{MS} values of enzymes at sub-lethal temperatures and different pressures. Furthermore, there were significant differences between the magnitudes of pressure on the MS inactivation ($p \leq 0.05$) for both enzymes in both juices, however, no statistically significant differences ($p > 0.05$) were found among the slopes of the regression lines for both enzymes in both juices (App. C). Hence, the effect of the pressure on the MS resistance would be the same regardless of the treatment media and the enzymes investigated. These results were agreement with the finding of López et al. (1994), Kuldiloke (2002), and Kuldiloke and Eshtiaghi (2009).

Table 3.15. Best fit equations calculated for describing the effect of pressure on the MS inactivation of PPO in treated apple juice and PME in treated orange juice.

Enzymes	Treatment Media	Amplitude (μm)	Pressure Linear Equation	R^2
PPO	Apple Juice	130.5	Log D_{MS} = - 0.002951 P + 1.342	0.99
PME	Orange Juice	130.5	Log D_{MS} = - 0.003308 P + 1.590	0.95

3.3.2.3 Relationship Between Power Measurements and MS Enzyme Inactivation

As previously mentioned in the microorganism inactivation part, Mañas et al. (2000b) reported that the lethality of US would rely on the amount of energy delivered into the treatment medium. Therefore, the rate of enzyme inactivation by MS would be determined by the energy transferred into the medium regardless of the combination of pressure and amplitude necessary to transfer that energy. In this investigation, the energy transmitted to the medium by ultrasonic waves at the different amplitudes (90,110, and 130.5 μm) and pressures (0,100, and 200 kPa) in the freshly squeezed two treatment media was calculated; and the relationship between the energy transmitted and the MS resistance of PPO and PME is shown in Figures 3.105 and 3.106.

Similar to microorganism inactivation, Figure 3.105 presented the effect of power input on the inactivation rates of PPO in apple juice and PME in orange juice. As mentioned before, PME in orange juice is more resistant than PPO in apple juice. It is seen that the D_{MS} values of PME inactivation have higher values than the D_{MS} values of PPO in apple juice at all conditions. The figure demonstrated these results.

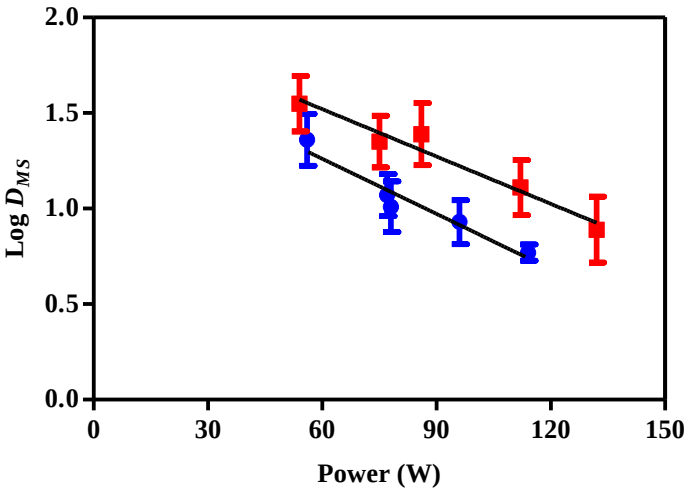

Figure 3.105. Effect of power input (W) on the inactivation rate (Log D_{MS}) of PPO (■) and PME (■) by US treated in apple and orange juices at different combinations of amplitudes (90, 110, and 130.5 μm) and pressures (0, 100, and 200 kPa).

It has been reported the existence of an exponential relationship between the Log_{10} D_{MS} values and the energy transmitted by ultrasound waves to the treatment medium, defining a z_{MS} value similar to that traditionally used when describing the kinetics of microbial inactivation by heat (Condón et al., 2011) and by MS treatment (Arroyo et al., 2011a). The treatment carried out at different combination of amplitudes (90, 110 and 130.5 μm) at a constant pressure (200 kPa), or of pressures (0,100 and 200 kPa) at a constant

amplitude (130.5 μm) in Figure 3.105. An exponential relationship between the D_{MS} values and the power delivered can be seen, with a z_{MS} mean value of 104 W for PPO in freshly squeezed apple juice and 121 W for PME in freshly squeezed orange juice, respectively. There were found no significant differences ($p > 0.05$) between the slopes of the regression lines for the both enzymes in both freshly squeezed fruit juices, and subsequently, between the z_{MS} values, obtaining a z_{MS} mean value of 113 W. According to these results, an increase in 113 W in the energy transferred into the freshly squeezed apple and orange juices by US will make the inactivation rate of PPO and PME increase by 10 times.

Figure 3.106 presented the relationship between the inactivation rates (Log D_{MS}) of PPO and PME and the energy transmitted by the ultrasound treated apple and orange juices. This graph allows us to compare the MS inactivation rate of both enzymes. As can be seen in the figure, it is more power necessary to inactivate PME in orange juice than to inactivate PPO in apple juice at the same conditions.

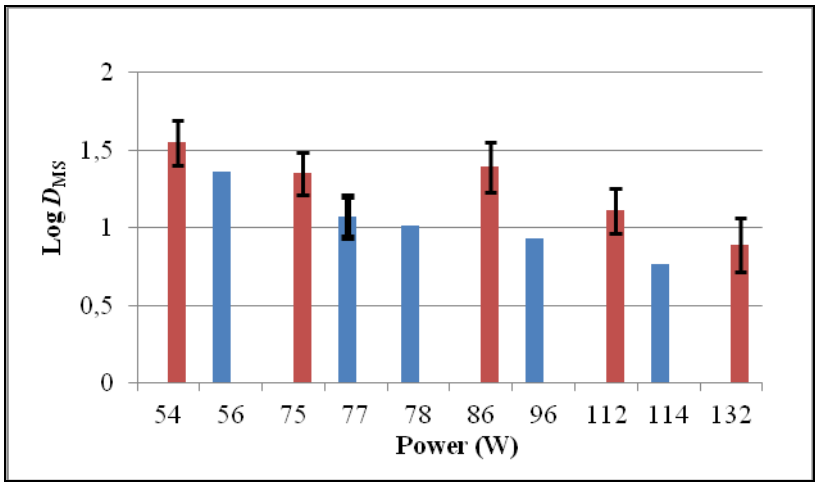

Figure 3.106. The relationship between the inactivation rates (Log D_{MS}) of PPO (blue bar) and PME (red bar) and the energy transmitted by the ultrasound treated apple and orange juices

3.3.3 Thermal Treatment

Thermal treatment is commonly used to inactivate enzymes. As similar to US, thermal treatments at same temperatures were performed to distinguish and evaluate between the effects of US and TT. Thermal treatments were carried out in a water bath at constant temperature as mentioned previously. Timing started after the samples reached the experimental temperature. According to many enzyme thermal inactivation studies (López and Burgos, 1995a, 1995b; López et al., 1997, 1998; Yemenicioğlu et al., 1997; Vercet et al., 1999, 2001, 2002a, 2002b; Kuldiloke, 2002; Raviyan et al., 2005; Tchoné et

al., 2005; Mañas et al., 2006; Soysal, 2008; Wu et al., 2008; Tefere et al., 2009), the inactivation of enzymes was more temperature dependent. Moreover, treatment temperature positively affected the inactivation rate of enzymes. Therefore, *D* values of thermal inactivation decreased as treatment temperature was increased.

Figure 3.107 shows the residual activities of PPO in freshly squeezed apple juice at different temperatures ranging from 25 to 60°C. The residual activity of PPO at 60 °C showed deviation from linearity because of heat-labile and heat-resistant iso-enzymes fractions of PPO. Many authors (Versteeg et al, 1978, 1980; Zawitowski et al., 1991; Oktay et al., 1995; Yemenicioglu et al., 1997; López et al., 1998; Vercet et al., 1999; Soysal, 2008; Murasaki-Aliberti et al., 2009) reported the different thermo-stability iso-enzymes of PPO in many fruit and vegetables. The temperature below 50 °C has no significant thermal inactivation effect on the PPO enzyme. Although the activity of PPO still remained around 25 % up the 50 °C temperature, above 50 °C the inactivation of PPO in apple juice affected positively. At the end of the treatment time (10 min), the inactivation of PPO was 74.38 % and 99.14 % for 50 and 60 °C, respectively. Tchoné et al. (2005) mentioned that PPO was not heat-stable enzyme and a short heating time of products between 70°C and 90°C was enough for PPO inactivation. Oktay et al. (1995) found that with raising temperature, PPO activity of Amasya apple in citrate-phosphate and HCl buffers decreased gradually. Moreover, in the study of Lee et al. (2009a), the residual activity of PPO in pineapples was measured as 20 U at 60 °C and PPO lost 83 % of its initial activity at 75 °C. The similar results mentioned from Yemenicioglu et al. (1997), Yemenicioglu (2002), Soysal (2008), and Kuldiloke and Eshtiaghi (2009), Fante and Noreña (2012). The increase in the temperature decreased the D_{TT} values of PPO as given in the Table 3.16 and there was statistically significant difference between *D* values and temperatures ($p \leq 0.05$). *z* values of PPO inactivation also were calculated 16.05 °C and given in the Table 3.16. Weemaes et al. (1998) reported that *z* value of PPO from grapes was 13.02 °C and Unal (2007) reported a *z* value of 14.04 °C for PPO of banana. The *z* value found in these studies compare well with the z values of 16 °C for canning ripe apricot and of 17.6 °C for green coconut water, reported by Heil et al. (1988) and Matsui et al. (2008), respectively.

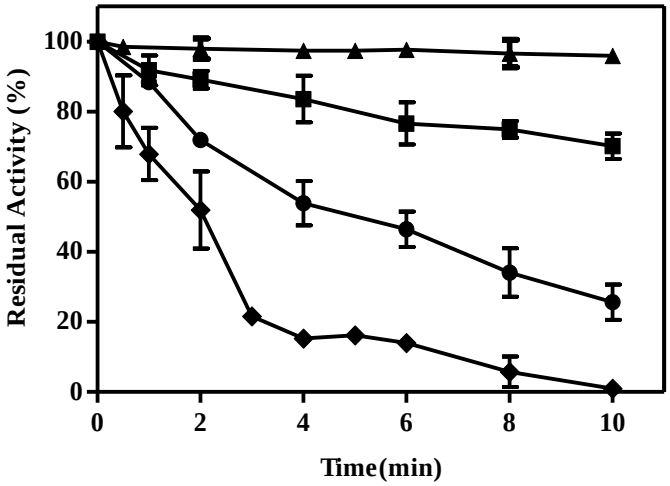

Figure 3.107. Residual activity of PPO in apple juice by TT at various temperatures [25 °C (control; ▲), 40 °C (■), 50 °C (●), and 60 °C (♦)].

As shown in the Figure 3.108, when PPO in squeezed apple juice was heat-treated, log residual activity decreased exponentially with treatment time. Because it has generally been associated with the presence of iso-enzymes of different thermal sensitivity (Versteeg et al, 1978, 1980; Vesteeg, 1979; Zawitowski et al., 1991; Oktay et al., 1995; Yemenicioglu et al., 1997; López et al., 1998; Vercet et al., 1999; Villamiel and Jong, 2000b; Anthon et al., 2002; Mañas et al., 2006; Soysal, 2008; Kuldiloke and Eshtiaghi, 2009; Murasaki-Aliberti et al., 2009), residual activity curves of thermal inactivation showing tails have been reported for PPO in different vegetables and fruits, such as garlic (*Allium sativum L.*) (Fante and Noreña, 2012), tomato (Kuldiloke and Eshtiaghi, 2009) and white yam (*Dioscorea rotundata*) (Eze et al., 2010). Although such behavior has been detected in only at 60 °C treatment in this investigation, the log residual plot of PPO at 60 °C showed linear line. It can be described the similar thermal resistance of PPO iso-enzymes in Golden Delicious apple juice (López et al., 1998). Therefore, the relationship between the log residual PPO activity and treatment time (min) in 25-60 °C temperature range followed first - order kinetics (Figure 3.108). The results of Gouzi et al. (2012) and Cheng et al. (2013) are in good agreement with this investigation. They reported that the PPO in aqueous mushroom extract followed a simple first-order inactivation kinetics.

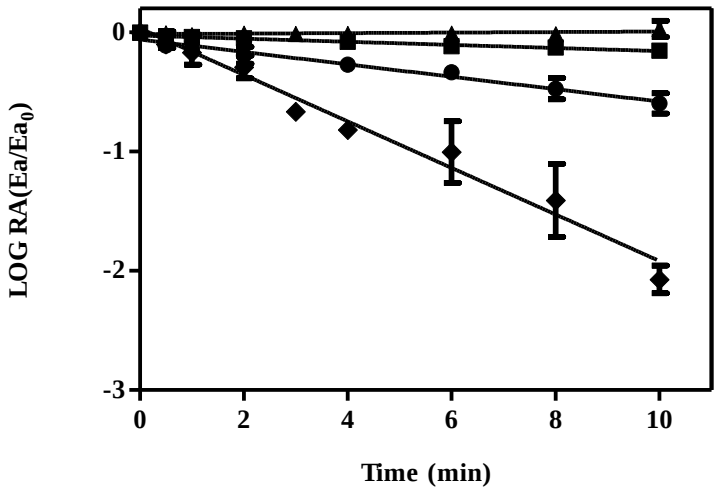

Figure 3.108. Inactivation rate of PPO in apple juice by TT at various temperatures [25 °C (control; ▲), 40 °C (■), 50 °C (●), and 60 °C (♦)].

The residual activity of PME in freshly squeezed orange juice *vs.* time (min) data obtained for temperatures of 25, 40, 50, 60, and 70 °C were plotted in the Figure 3.109. At 25 and 40 °C, PME lost approximately 3.5 % and 14 % of initial activity. However, the significant inactivation of orange juice PME was observed at the temperature higher than 50 °C. Terefe et al. (2009) also observed similar inactivation behavior for PME in tomato juice. At 70 °C, the inactivation of PME was 64.29 %. It is obtained that treatment temperature affected on the inactivation rate of PME. When the temperature was increased to 60 and 70 °C, the time needed to achieve 90 % reduction in PME residual activity was reduced to 36.83 and 23.33 min, respectively. Kuldiloke (2002) reported that D_{TT} values of citrus juice PE at 60 and 70 °C were 94.82 and 34.47 min, respectively. By the same approach, the D_{TT} of tomato PME were calculated 299.0 and 25.3 min at 61 and 72 °C, by Raviyan et al. (2005). Hence, the increase in the temperature decreased the D_{TT} values of PME as given in the Table 3.16. The significant differences were found between *D* values and temperatures ($p \leq 0.05$). *z* value of PME inactivation also was calculated 28.72 °C and given in the Table 3.16. At the thermal treatment temperature ranged from 62.5-86 °C in the study of López et al. (1998), *z* value of PME for tomato was estimated 16.93 °C. Versteeg et al. (1980) reported that z values of two isoenzymes of PE from Navel orange were estimated 6.5-11 °C from 60-70 °C, respectively. The *z* value found in this study compares well with the *z* value of 22.29 °C for tomato at 40-80 °C, reported by Kuldiloke and Eshtiaghi (2009).

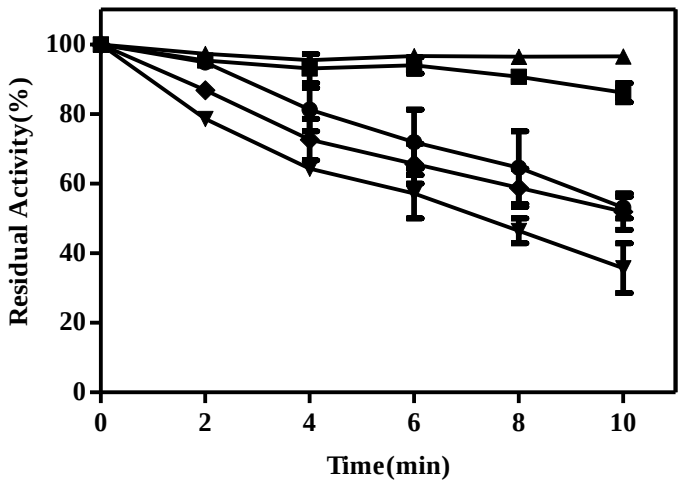

Figure.3.109. Residual activity of PME in orange juice by TT at various temperatures [25°C (control;▲), 40 °C (■), 50 °C (●), 60 °C (♦) and 70 °C (▼)].

The existence of various PME isoenzymes in orange juice with different degrees of thermo stability has also been reported (Vesteeg, 1979; Hou et al., 1997; Van Den Broeck et al., 1999; Vercet et al., 1999). Nevertheless, log-plot of residual activity *vs.* treatment time (min) fitted with first-order kinetic in the experiments described here (Figure 3.110). The similar thermo-resistance of PME isoenzymes can explain this case in this investigation (López et al., 1998). Similar to studies of López and Burgos (1995a, 1995b), López et al. (1998), Raviyan et al. (2005), Wu et al. (2008), Yaldagard et al. (2008), Terefe et al. (2009), Ercan and Soysal (2011), and Cheng et al. (2013), increasing the temperature increased the inactivation rate.

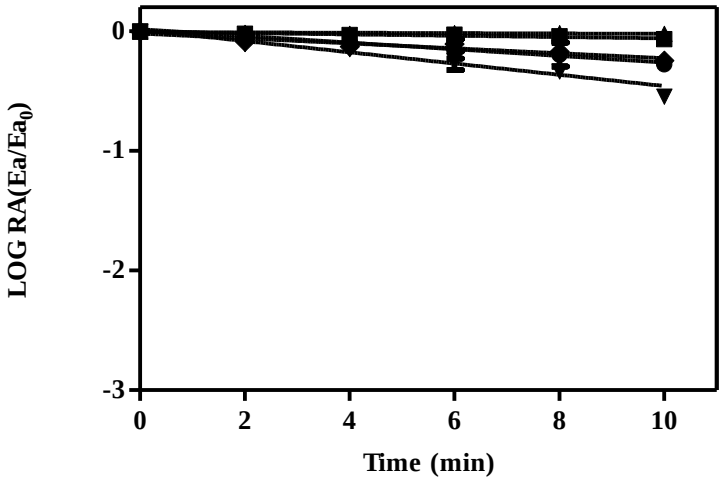

Figure 3.110. Inactivation rate of PME in orange juice by TT at various temperatures [25°C (control;▲), 40 °C (■), 50 °C (●), 60 °C (♦) and 70 °C (▼)].

Table 3.16. Resistance parameters (D_{TT}) of PPO in apple juice and PME in orange juice to ultrasound treatments. ANOVA test ($p \leq 0.05$) was conducted within each group, as shown in Significant level column. To identify intergroup differences after significant differences in the ANOVA test, multiple comparisons were performed using Tukey's test. Values with the same superscript did not show statistically significant differences ($p > 0.05$).

Enzymes	Treatment Media	pH	Temperature (^{o}C)	Fit Parameters			
				D_{TT} mean (min)	$SD(\pm)$	R^2	Significant Level
PPO	Apple Juice	3.4	25	829.03	157.88	0.10	$p \leq 0.05$
			40	69.01	8.15	0.73	
			50	19.85	0.69	0.90	
			60	5.47	0.36	0.95	
			70	NC	-	-	
			z value	16.05 °C			
PME	Orange Juice	3.7	25	861.07	24.50	0.48	$p \leq 0.05$
			40	192.80	59.55	0.67	
			50	48.96	6.34	0.83	
			60	36.83	7.27	0.83	
			70	23.33	4.26	0.91	
			z value	28.72 °C			

In the literature, it is shown that there are some significant differences between the reported thermal inactivation parameters (*D* and *z* values) of PPO and PME (Versteeg et al., 1980; López et al., 1994; Oktay et al., 1995; Anthon et al., 2002; Yemenicioglu and Cemeroglu, 2003; Raviyan et al., 2005; Unal, 2007; Matsui et al., 2008; Soysal, 2008; Wu et al., 2008; Kuldiloke and Eshtiaghi, 2009; Lee et al., 2009a; Murasaki-Aliberti et al., 2009). For example, Yemenicioglu et al. (1997) reported D_{TT} values of 30.3-56.6 min at 73 °C and 8.1-14.4 min at 78°C for various apple cultivars PPO. Murasaki-Aliberti et al. (2009) investigated that D_{TT} value determined in the range from 6 s to 11.3 min at 86.9°C for heat labile and resistant fraction of green coconut water PPO. Our data also fits these values (D_{TT}= 5.47 min at 60 °C). The reported *z* values for the thermal inactivation of PPO in many fruits and vegetables vary from 4.5 to 46.73 °C (Heil et al., 1988; López et al., 1994; Oktay et al., 1995; Yemenicioglu et al., 1997; Weemaes et al., 1998; Chutintrasri and Noomhorm, 2006; Unal, 2007; Matsui et al., 2008; Kuldiloke and Esthiaghi, 2009; Murasaki-Aliberti et al., 2009; Cheng et al., 2013). Our *z* value of 16.05 °C is in close agreement with the value of Heil et al. (1988) for apricot PPO and Matsui et al. (2008) for green coconut water PPO who reported *z* values of 16 and 17.6 °C, respectively. Moreover, Vercet et al. (1999) obtained D_{TT} values of PME in orange juice were showed to differ among the 7.2-500 min from 72 to 82 °C. Raviyan et al. (2005) reported D_{TT} values of tomato PME at 70 °C in the ranging from 1.5 to 36.5 min. Our D_{TT} value of PME at same temperature (D_{TT} = 23.33 min) falls within these ranges. The *z* values for thermal inactivation of PME in many fruits and vegetables were reported as 4.1 to 47 °C (López et al., 1994, 1998; Vercet et al., 1999; De Gennaro et al, 1999; Anthon et al., 2002; Raviyan et al., 2005; Wu et al., 2008; Terefe et al, 2009; Ercan and Soysal, 2011). Our z value of 28.72 °C was found in the range of these results. Several factors may be responsible for the observed discrepancies in inactivation kinetic parameters (Terefe et al., 2009). Some of these studies were related to inactivation of enzymes in many varieties of fruits and vegetables (Heil et al., 1988; De Gennaro et al., 1999; Vercet et al., 2002a, 2002b; Unal, 2007; Wu et al., 2008; Ganjloo et al., 2009), whilst others were related to purification and investigation of characteristics of enzymes (Oktay et al., 1995; Yemenicioğlu et al., 1997; López et al., 1998; Vercet et al., 1999; Raviyan et al., 2005; Ganjloo et al., 2009; Kuldiloke and Eshtiaghi, 2009; Murasaki-Aliberti et al., 2009; Terefe et al., 2009; Ercan and Soysal, 2011; Cheng et al., 2013). Whitaker (1972) mentioned that an enzyme less stable in its purified form than in an intact tissue or in homogenate or in food matrix, because it is protected by the presence of other materials, such as proteins, carbohydrates, and pectins. Vercet et al. (1999) also found that the heat resistance of PME enzyme was higher in the orange juice than in citrate buffer.

In literature, the other reason of difference between the kinetic parameters of enzymes can be related that various isoenzymes have different degrees of thermo stability in various fruits and vegetables as reported (Versteeg et al, 1978, 1980; Zawitowski et al., 1991; Yemenicioglu et al., 1997; López et al., 1998; Vercet et al., 1999; Soysal, 2008; Murasaki-Aliberti et al., 2009). PPO is generally considered an enzyme of low thermo stability. Marangoni (2003) reported that PPO enzyme was less thermo stable at higher temperatures. The study of Zawitowski et al. (1991) has shown that the heat stability varied between cultivars and multiple form of PPO from the same source, as well as between fruit tissue homogenates and their respective juices. Soysal (2008) and Murasaki-Aliberti et al. (2009) also mentioned that heat labile and heat resistant fractions

of PPO in apple cultivars and Yemenicioglu et al. (1997) reported heat stability of apple PPO varies with the six variety (Golden Delicious, Starking Delicious, Granny Smith, Gloster, Starcrimson, and Amasya). In the same perspective, the high heat stability of PME has mentioned by many researchers (Versteeg et al, 1978, 1980; Snir et al., 1996; López et al., 1998; Vercet et al., 1999). Van Den Broeck et al. (1999) reported that thermo stable PE from Navel orange appeared to be less thermo stable than the counterpart in Valencia orange. At least five forms of PE in Navel orange (Versteeg, 1979) and the extraction of thermo labile and thermo stable isoforms of PE from Valencia oranges (Hou et al., 1997) were reported. A thermo resistant isoenzyme of PME generates a fraction of the total PME activity that varies with cultivar, degree of ripening, etc. (Versteeg et al., 1980; Snir et al., 1996; Vercet et al., 1999). From the Figure 3.111 as can be seen that PPO was relatively more heat sensitive than PME. When the temperature was increased from 25 to 60 °C (for PPO) or to 70 °C (for PME) in the thermal treatment, PPO enzyme lost almost 98% of initial activity but PME activity declined slowly and it still needed to time for 90% achieve thermal inactivation at studied temperature condition.

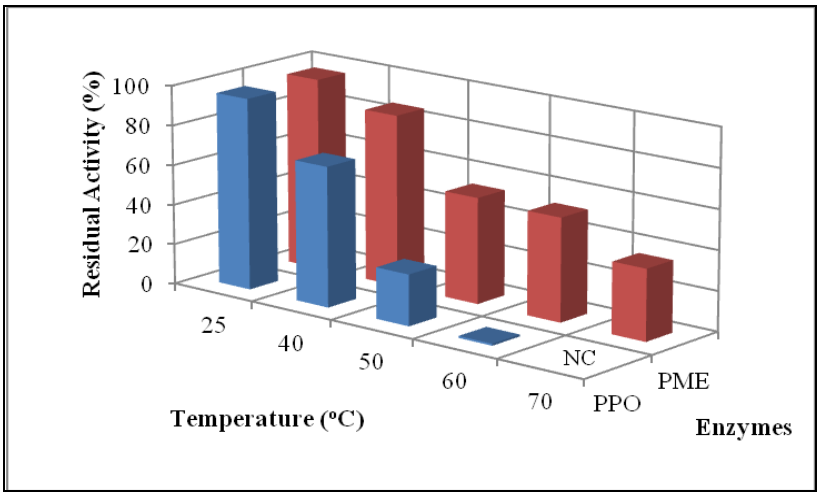

Figure 3.111. Effect of temperature on residual activity(%) of PPO in apple juice (blue bar) and PME in orange juice (red bar) at different temperatures (25, 40, 50, 60 and 70°C).

The plot for Log *D* of thermal treatment of PPO and PME at different temperatures are presented in the Figure 3.112. As can be seen in the figure, Log D_{TT} vs. temperature was apparently linear, which was shown to follow the first order kinetic model. The estimated kinetic parameters, *z* values, are presented in Table 3.16. In general, the many reported thermal treatment studies have shown the first order kinetic model (López et al., 1994, 1998; López and Burgos, 1995a, 1995b; De Gennaro et al., 1999; Kuldiloke, 2002; Raviyan et al., 2005; Soysal, 2008; Tiwari et al., 2008; Wu et al., 2008; Terefe et al.,

2009; Ercan and Soysal, 2011; Cheng et al., 2013) and the results coincide with the our results.

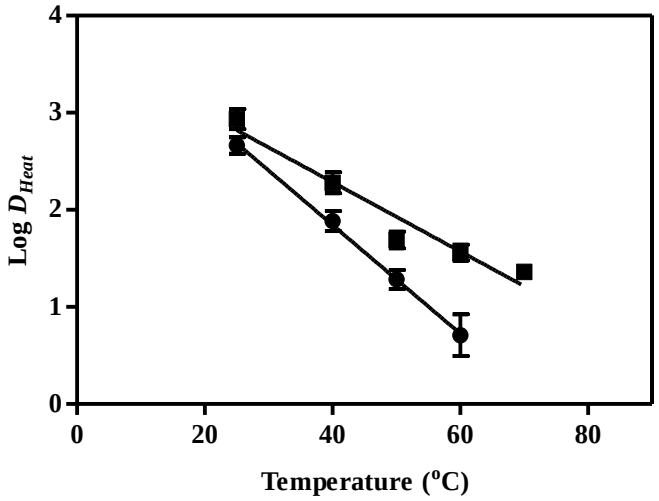

Figure 3.112. Log D_{Heat} values of PPO (●) in apple juice and PME (■) in orange juice of TT inactivation.

3.3.4 MTS Experiments

3.3.4.1 Effects of Temperature on Inactivation of Enzymes

For stabilization and long shelf life of some food products, enzymes must be inactivated or their activity reduced. This is the driving force for the increased interest in an alternative method of inactivation: US. The effects of ultrasonic waves on enzymes are very complex. Sensitivity to US depends on the conditions of the treatment (McClements, 1995) as well as on the nature of the enzyme (Sala et al., 1995). In some cases, US treatments alone do not inactivate the enzymes and therefore, it is necessary additive helping effect, heat and pressure, for inactivation (Earnshaw et al., 1995; Sala et al., 1995; Villamiel and Jong, 2000b). MTS is a technology combining efficiently the inactivating effect of heat and ultrasonic waves under pressure. The effect of MTS on many enzymes has been studied in different laboratory systems (López et al., 1994, 1998; López and Burgos, 1995a, 1995b; Vercet at al., 1997, 2001, 2002a, 2002b; Villamiel and Jong, 2000b; Kuldiloke, 2002; Mañas et al., 2006; Kuldiloke and Eshtiaghi, 2009; Cheng et al., 2013).

In this work, the inactivation of PPO and PME by US under pressure at different temperatures (50 and 60 °C) in apple and orange juices was studied. MTS treatments performed at different combination of pressures (100 and 200 kPa) and temperatures (50 and 60°C) at constant amplitude (110 μm) in both juices. The curves for US (20 kHz, 110

μm) under two pressures-induced inactivation of PPO in apple juice at different temperatures are presented in Figure 3.113. As can be seen from the figure, the activity of PPO in apple juice decreased under MTS at different pressure-temperature combinations. Kuldiloke (2002) mentioned that the MTS inactivation effect increase with increasing pressure and temperature.

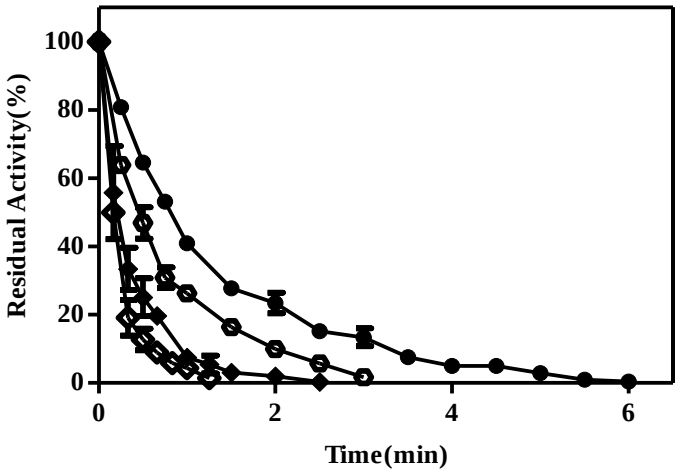

Figure 3.113. Effect of temperature on residual activity of PPO in apple juice by MTS (110 μm) under different pressure-temperature combinations [100 kPa, 50 °C (●); 100 kPa, 60 °C (♦); 200 kPa, 50°C (○); and 200 kPa, 60 °C (◊)].

The MTS inactivation of PPO at different pressure-temperature combinations is presented in Figure 3.113. Under the same pressure the increasing temperature, the D_{MTS} values of PPO decreased (Table 3.17). At 200 kPa, the D_{MTS} values of PPO are 1.88 and 0.72 min for 50 and 60 °C, respectively. In the same way, the D_{MTS} values of PPO at constant 100 kPa pressure are 3.27 and 1.09 min for 50 and 60 °C, respectively. Same phenomenon seems to be common in the literature for different enzymes (López et al., 1998; López and Burgos, 1995a, 1995b; Vercet et al., 1997, 1999, 2001; Kuldiloke, 2002; Mañas et al., 2006; Wu et al., 2008; Yaldagard et al, 2008 Terefe et al., 2009). In addition to that the increasing pressure also affected positively the inactivation rate, hence D_{MTS} values decreased. At 60 °C, the D_{MTS} values of PPO are 1.07 and 0.72 min for 100 and 200 kPa, respectively. According to studies of Kuldiloke (2002) and Kuldiloke and Eshtiaghi (2009), increasing the enzyme MTS inactivation (PPO, POD, PME, and PG) in tomato juice occurred with increasing of pressure (0-400 kPa). The combination of 200 kPa pressure - 60°C temperature showed the higher inactivation rate of PPO in freshly squeezed apple juice. D_{MTS} values of PPO were given in the Table 3. 17. Statistical analysis shows that there was significant difference between D_{MTS} values of PPO (p<0.05). In contrast to MS and thermal treatment alone, MTS caused significant inactivation of apple juice PPO at all studied temperature and pressure combinations. The comparison of the D values of MS (200 kPa, 110 μm, 35°C), TT (60°C) and MTS (200

kPa, 110 µm, 60°C) allows determining the effective treatment to inactivate PPO. *D* value of MTS (D_{MTS}= 0.72 min) is smaller than D_{MS} (D_{MS}= 8.46 min) and than D_{TT} (D_{TT}= 5.13 min). These results presented here show that MTS has higher inactivation efficiency with respect to apple juice PPO than TT and MS. López et al. (1994), Kuldiloke (2002), Kuldiloke and Eshtiaghi (2009) and Cheng et al. (2013) worked on PPO inactivation by MTS and they found also similar observation to our results.

Figure 3.114 is plotted with the four different combinations of pressure - temperature at constant amplitude (110 µm). Log residual PPO activity versus MTS treatment time (min) graph is exhibited the kinetics of MTS inactivation. Some studies were mentioned biphasic reaction kinetics such as Kuldiloke and Eshtiaghi (2009) for tomato PPO. Although the biphasic behavior was perceived from the Figure 3.113 because of presence of different thermo-stability iso-enzymes of PPO in apple juice (Oktay et al., 1995, Yemenicioğlu et al., 1997), as seen from the Figure 3.114, the linear lines with high correlation coefficients (0.97 to 0.99) fit well to an apparent first order model, because of the PPO iso-enzymes may have similar MTS resistance (López et al., 1998). Therefore, our results are in agreement with Cheng et al. (2013) and López et al. (1994) for mushroom PPO. Other authors (López et al., 1994, 1998; López and Burgos, 1995a, 1995b; De Gennaro et al., 1999; Vercet et al., 1999, 2001; Raviyan et al., 2005, Wu et al., 2008; Yaldagard et al., 2008; Terefe et al., 2009; Ercan and Soysal, 2011; Cheng et al., 2013) reported the linear regression model of enzyme inactivation by ultrasonication.

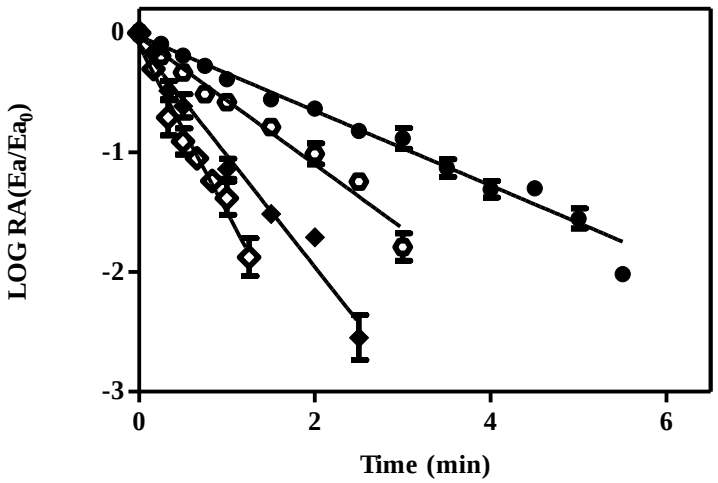

Figure 3.114. Inactivation rate of PPO in apple juice by MTS (110 µm) under different pressure-temperature combinations [100 kPa, 50 °C (●); 100 kPa, 60 °C (♦); 200 kPa, 50°C (○); and 200 kPa, 60 °C (◊)].

The residual activity of PME in orange juice by MTS (200 kPa, 110 µm) was investigated for two different temperatures in the Figure 3.115. Similar to PPO, the inactivation rate of

PME increased with increase in the temperature under constant pressure (200 kPa). Kuldiloke (2002) reported that when the temperature increased from 40 to 90°C at 400 kPa, the MTS inactivation of PME increased in tomato juice.

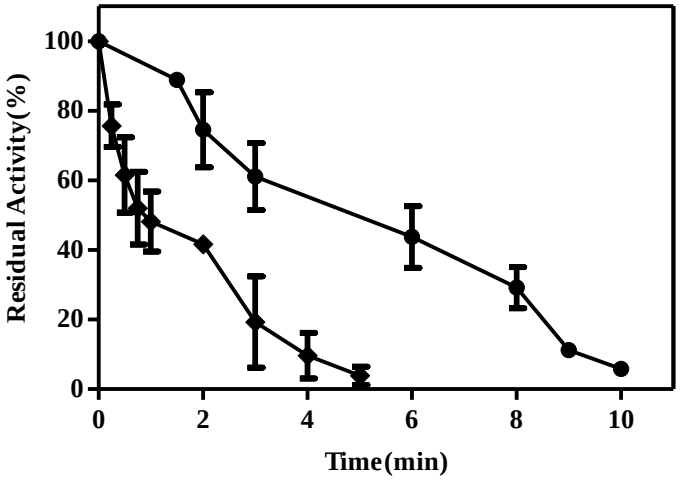

Figure 3.115. Effect of temperature on residual activity of PME in orange juice by MTS (200 kPa, 110 µm) at different temperatures [50 °C (●) and 60 °C (♦)].

The MTS inactivation of PME at different temperatures is presented in Figure 3.116. The inactivation kinetics of orange juice PME by MTS is well described by first order inactivation kinetics, similarly PPO inactivation. Our results are in agreement with López et al. (1994) and De Gennaro et al. (1999) for horseradish PE in a model solution, Cruz et al. (2006) for water cress PE, and Raviyan et al. (2005) and Terefe et al. (2009) for tomato PME. It is also appears that both simple and biphasic reaction kinetics equations are described as US inactivation kinetic model for different source of PME such as for tomato (Kuldiloke, 2002; Kuldiloke and Eshtiaghi, 2009) and orange juice (Vercet et al., 1999; Tiwari et al., 2009). Although deviation from the linearity of the residual activity graphs of PME was observed in this investigation (Figure 3.115), first order inactivation kinetic model well fitted with the plot of log residual activity. It can be explained the similar MTS resistance of PME isoenzymes in Navelina orange juices (López et al., 1998). Under the same pressure the increasing temperature, the D_{MTS} values of PME in orange juice decreased. At 200 kPa pressure, the decimal reduction time values (D_{MTS}) were calculated 13.68 and 4.07 min for 50 and 60°C, respectively. Such decrease in the efficacy of US with increase in temperature has also been observed by López and Burgos (1995a, 1995b); Vercet et al. (1997, 1999, 2001); López et al. (1998); Kuldiloke (2002); Mañas et al. (2006); Wu et al. (2008); Yaldagard et al. (2008); and Kuldiloke and Eshtiaghi (2009). Terefe et al. (2009) found that the reduction of *D*-value of tomato PME decreased to 1.5 times at 75 °C compared to 6 times at 60 °C. D_{MTS} values of PME were given in the Table 3.17. Significant difference was observed between D_{MTS} values ($p < 0.05$). The *D*-value obtained from MTS at any observed temperature was much smaller

than those for thermal (D_{50^oC} = 48.42 min and D_{60^oC} = 35.90 min) and MS (D_{MS} = 12.06 min) inactivation. Different authors (López et al., 1994; Vercet et al., 1999, 2002a; Kuldiloke, 2002; Raviyan et al., 2005) have studied MTS inactivation of PME enzyme in laboratory media and they were mentioned that MTS was an effective enzyme inactivation technique.

PME was treated by MTS (200 kPa, 110 μm) at 50 and 60 °C in freshly squeezed orange juice to observe the kinetic model of MTS treatment on the enzyme inactivation. The results of these experiments are shown in the Figure 3.116, where the MTS resistances of PME with the linear lines fit well to a first – order kinetic model. The rate of PME inactivation by MTS is at 60 °C higher than at 50 °C in orange juice. The kinetic model of PME inactivation by MTS is compared with the data collected by other authors such as López et al. (1998), Vercet et al. (1999), Raviyan et al. (2005), Wu et al. (2008), and Terefe et al. (2009) for tomato PME; Ercan and Soysal (2011) for tomato PE; López et al. (1994) and De Gennaro et al. (1999) for horseradish PE and these results are coincide with our results.

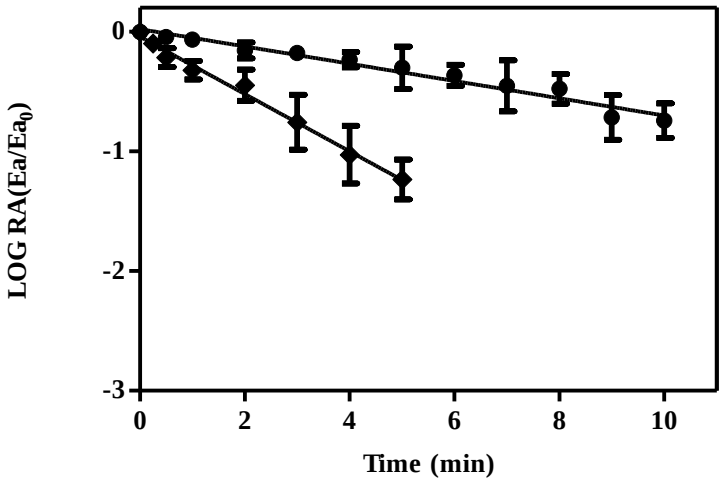

Figure 3.116. Inactivation rate of PME in orange juice by MTS (200 kPa, 110 μm) at different temperatures [50 °C (●) and 60 °C (♦)].

Table 3.17. Resistance parameters (D_{MTS}) of PPO in apple juice and PME in orange juice to ultrasound treatments. ANOVA test ($p \leq 0.05$) was conducted within each group, as shown in Significant level column. To identify intergroup differences after significant differences in the ANOVA test, multiple comparisons were performed using Tukey's test. Values with the same superscript did not show statistically significant differences ($p > 0.05$).

Enzymes	Treatment media	Ampitude (μm)	Pressure (kPa)	Temperature (^{o}C)	Fit Parameters			
					D_{MTS} mean (min)	$SD(\pm)$	R^2	Significant Level
PPO	Apple Juice	110	100	50	3.269	0.220	0.99	$p \leq 0.05$
				60	1.093	0.086	0.97	
			200	50	1.880	0.103	0.97	
				60	0.721	0.049	0.97	
PME	Orange Juice	110	200	50	13.683	2.448	0.85	$p \leq 0.05$
				60	4.067	0.512	0.96	

The effect of temperature on the inactivation rates of PPO in apple juice and PME in orange juice by MTS treatment (110 µm, 220 kPa) at three different temperatures (35, 50, and 60 °C) is plotted in the Figure 3.117. As can be seen from the figure, raising the treatment temperature of the combined treatment from 35 °C to 60 °C caused an 12-fold decrease in the *D* value of PPO in apple juice, and an 3-fold decrease in the *D* value of PME in orange juice. At the end of the MTS treatment time, less than 3% of the initial activity was detected for PPO in apple juice and for PME in orange juice at 60 °C. The inactivating effect of MTS increased with temperature. Mañas et al. (2006) mentioned same observation for egg white lysozyme in phosphate buffer by MTS (200 kPa, 117 µm, 20 kHz) at different temperatures. Similar results were reported by Kuldiloke (2002) regarding the inactivation of tomato PPO and PME by MTS (400 kPa, 24 kHz, 35 µm) at range from 40 to 90 °C.

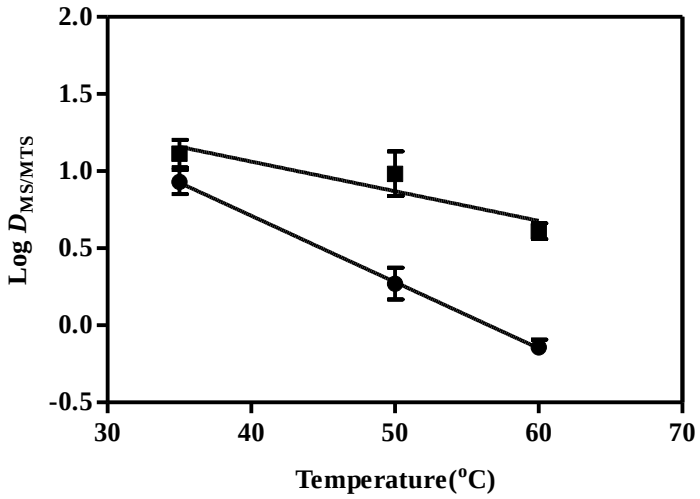

Figure 3.117. Effect of temperature on the inactivation rates of PPO (●) in apple juice and PME (■) in orange juice by MTS treatment (110 µm, 200 kPa) at three different temperatures (35, 50, and 60 °C).

To compare the inactivation of thermal and MS/MTS treatments, the graphs of the log *D* *vs.* temperature (°C) were plotted in the Figure 3.118 for PPO in apple juice and in the Figure 3.119 for PME in orange juice. At 50 and 60 °C manothermosonicated fruit juices contained residual activities of PPO and PME that were significantly lower than the thermally treated fruit juices at the same temperatures. The *D* values of thermally treated samples decreased from 69.01 min to 5.47 min for PPO and from 192.80 min to 36.83 min for PME with the increase of temperature from 40 to 60 °C, respectively. In same way, the application of MTS markedly decreased the *D* values from 8.46 min to 0.72 min for PPO and from 12.98 min to 4.067 min for PME with the increase of temperature from 35 to 60 °C, respectively. The efficiency of MTS, expressed as the ratio $D_{60°C}$ of thermal treatment / $D_{60°C}$ of MTS, is ~ 7.6 for PPO in apple juice and ~ 9.1 for PME in orange

juice. The similar results obtained in this investigation were observed by Vercet et al. (1999); Wu et al. (2008); Kuldiloke and Eshtiaghi (2009). Kuldiloke and Eshtiaghi (2009) found that the D_{MTS} values of tomato PPO and PME inactivation at constant amplitude (35 μm) decreased from 25.26 min to 0.96 min for PPO and from 29.62 min to 0.29 min for PME a when temperature increased from 40 to 90°C. Moreover, Wu et al. (2008) worked on TS (75 μm) inactivation of tomato PME at the different temperatures (55-70 °C). Thermal and MS/MTS inactivation of PPO in apple juice and PME in orange juice showed apparent first order kinetics (Figures 3.118 and 3.119). Other authors reported similar observation for thermal inactivation (López et al., 1994, 1998; López and Burgos, 1995a, 1995b; De Gennaro et al., 1999; Kuldiloke, 2002; Raviyan et al., 2005; Tiwari et al., 2008; Soysal, 2008; Wu et al., 2008; Terefe et al., 2009; Ercan and Soysal, 2011; Cheng et al., 2013) and US inactivation (López et al., 1994, 1998; López and Burgos, 1995a, 1995b; De Gennaro et al., 1999; Vercet et al., 1999, 2001; Raviyan et al., 2005; Cruz et al., 2006; Mañas et al., 2006; Wu et al., 2008; Yaldagard et al., 2008; Terefe et al., 2009; Ercan and Soysal, 2011; Cheng et al., 2013).

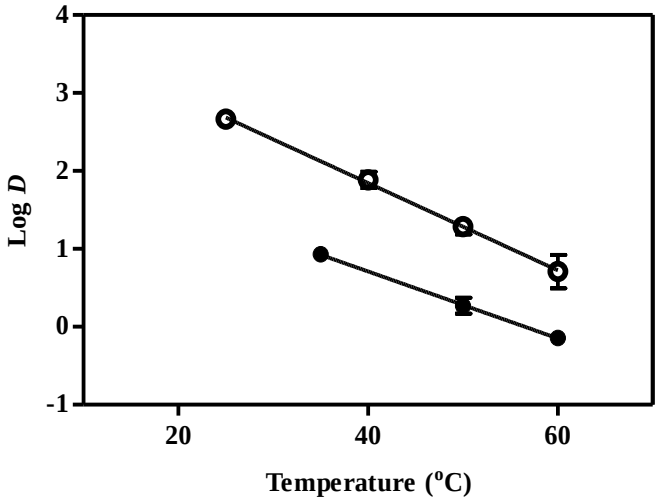

Figure 3.118. Effect of temperature on PPO inactivation by heat (o) and MS/MTS (●).

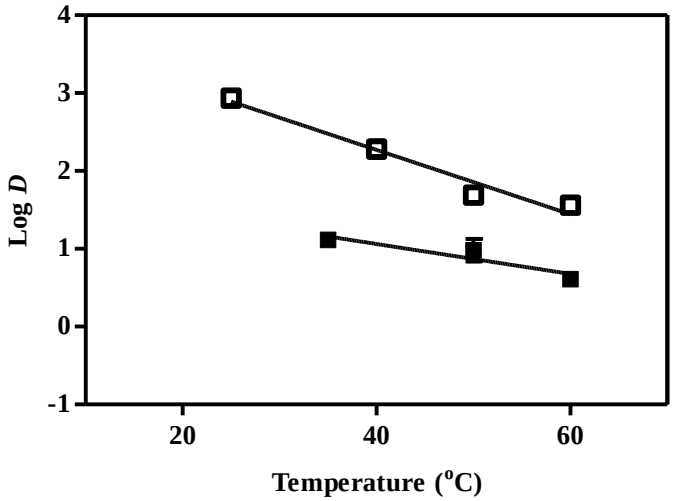

Figure 3.119. Effect of temperature on PME inactivation by heat (□) and MS/MTS (■)

The comparison of the inactivation by thermal treatment with the inactivation by US treatment shows that the combined process (MTS) were more efficient on reducing the enzyme activity than thermal treatment acting alone in Figures 3.120, 3.121, 3.122 and 3.123. As an illustration, whereas 5.47 and 36.38 min are needed under a heat treatment at 60 °C for inactivating 90 % of the PPO in apple juice and PME in orange juice, respectively, the same level of the inactivation might be achieved after 0.72 and 4.06 min of MTS treatments at the same temperature. Although it is generally expected that the combined processes (MS and MTS) were more efficient on the reducing the enzyme activity than heat acting alone, MS treatment was less effective on the inactivation of PPO enzyme than heat and MTS (Figures 3.120 and 3.121). Because PPO is considered the heat labile enzyme in literature (Zawitowski et al., 1991; Yemenicioglu et al., 1997; Marangoni, 2003; Soysal, 2008; Murasaki-Aliberti et al., 2009), heat inactivation was more effective on the inactivation of PPO in apple juice after 2 min treatment. Contrary to, MS and MTS process were more effective on the inactivation of PME in orange juice than on the inactivation of heat (Figures 3.122 and 3.123), because of the high thermo stability of PME (Vesteeg, 1979; Anthon et al., 2002).

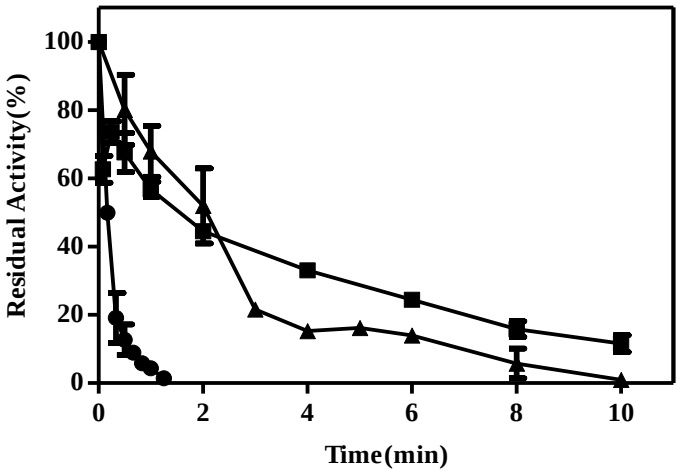

Figure 3.120. Residual activity of PPO subjected to heat (60 C) (▲), MS (35 C, 200 kPa, 110 μm) (■), and MTS (60 C, 200 kPa, 110 μm) (●) treatments in apple juice.

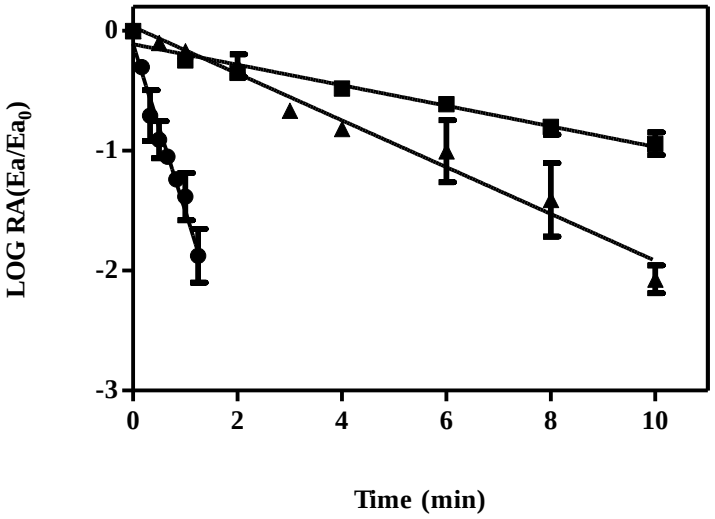

Figure 3.121. Inactivation rate of PPO subjected to heat (60 C) (▲), MS (35 C, 200 kPa, 110 μm) (■), and MTS (60 C, 200 kPa, 110 μm) (●) treatments in apple juice.

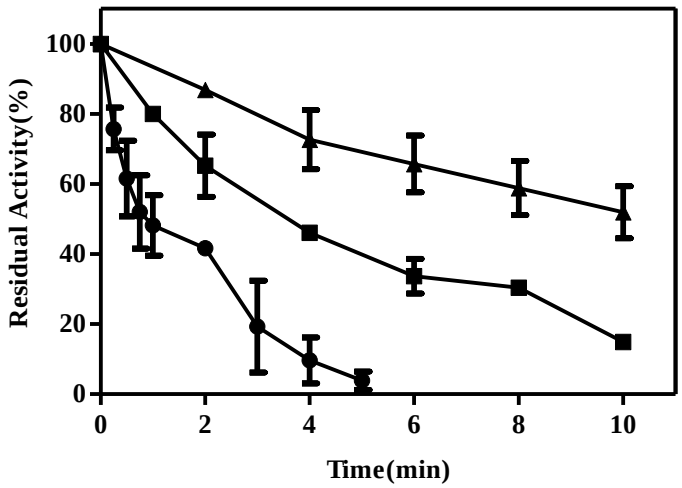

Figure 3.122. Residual activity of PME subjected to heat (60 C) (▲), MS (35 C, 200 kPa, 110 μm) (■), and MTS (60 C, 200 kPa, 110 μm) (●) treatments in orange juice.

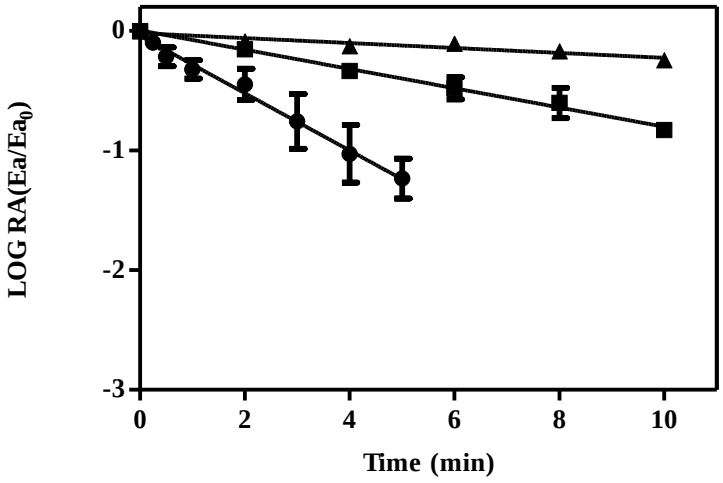

Figure 3.123. Inactivation rate of PME subjected to heat (60 C) (▲), MS (35 C, 200 kPa, 110 μm) (■), and MTS (60 C, 200 kPa, 110 μm) (●) treatments in orange juice.

As can be seen from the Figure 3.124, the both enzymes studied do not present the same sensitivities to ultrasonic inactivation. The ultrasonic resistance of PME in orange juice was much higher than PPO in apple juice. For example, at 200 kPa, D values of PPO in apple juice were smaller than *D* values of PME in orange juice. The difference between the US sensitivities of enzymes can be related to different structure of enzyme active centers and to susceptibility of these active centers to heating inactivation (López et al., 1994, 1998; Villamiel and Jong, 2000b; Kuldiloke, 2002; Terefe et al., 2009). Additionally of this reason, other factor may be the increase in the temperature diminishes the viscosity of the liquid medium favors the penetration of the waves (Earnshaw et al., 1995), and decreases the violence of the implosion of the formed bubbles (Sala et al., 1995). Vercet et al. (2002a) mentioned that the much higher viscosity of tomato juice compared to aqueous buffer caused less cavitation effect in the juice compared to the buffer and less pronounced effect of US on the enzyme. Another important factor is that the shear stress and turbulence produced by the effect of US (Price, 1990) can also be responsible for these differences. The different components of the sonication medium may also have a protective effect on the enzyme (Whitaker, 1972). Generally, some authors have demonstrated that the effect of US waves increases with solid concentration (Santamaria and Castellani, 1952; Berliner, 1984; Sala et al., 1995) and diminished with increasing enzyme concentration (Raharintsoa et al., 1977; Sala et al., 1995). Similarly, microorganism experiments, enzyme may be protected or easily inactivated by other components of acidic fruit juice. As explained in the Introduction section, apple and orange juices have different chemical structure. Vercet et al. (1999) reported the inactivation of PME in orange juice and in buffer (pH 3.5) by MTS (20 kHz, 117 μm, 200 kPa, 72 ^{o}C). The *D* value of PME in buffer was smaller than that in orange juice. The author explained that this difference depends on the orange juice components (pectin, proteins, etc...) which were responsible for the PME protection against MTS inactivation.

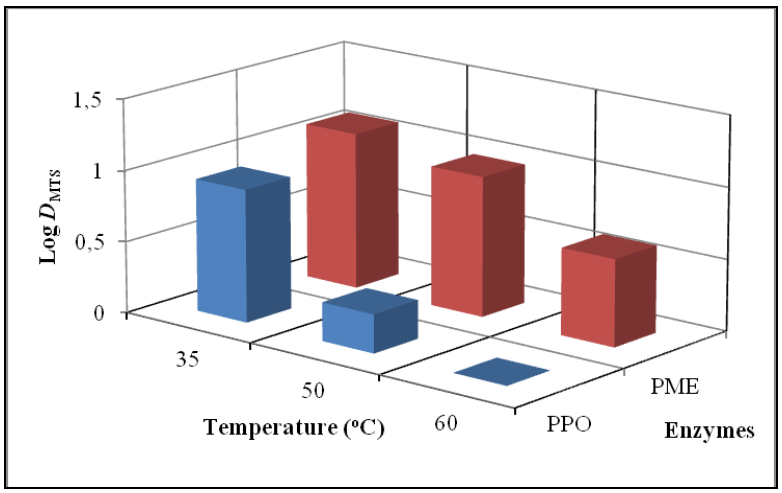

Figure 3.124. Inactivation rates of PPO (blue bar) in apple juice and PME (red bar) in orange juice by MTS treatment (110 µm, 200 kPa) for three different temperatures (35, 50, and 60°C).

Synergies between heat and ultrasound have been reported for enzyme inactivation of different enzymes such as PPO (López et al., 1994; Kuldiloke , 2002; Cheng et al., 2013), PME (López et al., 1994, 1998; Vercet et al., 1999; Kuldiloke, 2002; Raviyan et al., 2005; Wu et al., 2008; Terefe et al., 2009), PE (Kuldiloke, 2002; Ganjloo et al., 2009; Ercan and Soysal, 2011), PG (López et al., 1998; Terefe et al., 2009), native enzymes of milk (Villamiel and Jong, 2000b), lysozyme (Mañas et al., 2006). Therefore, and in order to determine whether this increase in inactivation by MTS processes over heat processes was due to an additive effect or to a synergistic effect, the experimental D_{MTS} values were compared with their corresponding theoretical D_{MTS} values (Table 3.18). According to the results here, the D values obtained from MTS at 50 and 60 °C were much smaller than those for thermal inactivation. These results also indicate that the combination of thermal and ultrasound under pressure treatment has synergistic effect on the inactivation of PPO in apple juice and of PME in orange juice. The maximum synergism was observed when inactivating PME in orange juice by MTS at 60°C, the experimental D_{MTS} value was an 89 % lower than the theoretical D_{MTS} corresponding to the sum of the effectiveness of heat and MS treatment acting simultaneously but independently.

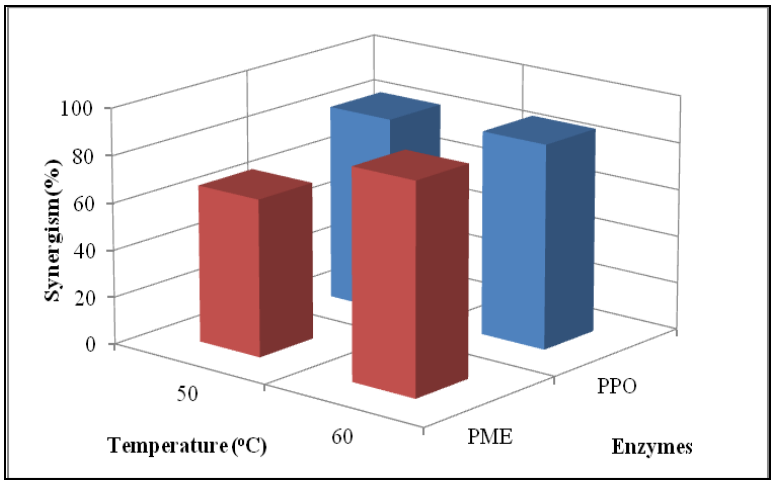

Figure 3.125. The synergistic effect of the combined MTS treatment on inactivation of PPO in apple juice (blue bar) and PME in orange juice (red bar) at different temperatures (50 and 60 °C).

To observe the increasing of synergistic effect of the combined MTS treatment with increasing of temperature, the synergism (%) *vs.* temperature (°C) graph was plotted in Figure 3.125. According to the Figure 3.125, the synergism of MTS on the inactivation of PPO and PME increased when temperature increased. For both enzymes, the synergistic effect has almost same percentage and very close to 100 %. Therefore, MTS can be very effective enzyme inactivation method in laboratory studies. It has been found that MTS could reduce the intensity (time and/or temperature) of the heat treatment used for orange juice stability and apple juice browning. For pectic and PPO enzymes inactivation by MTS has lower *D* values than by simple heating. In order to analyze the mechanisms of ultrasonic inactivation, there are several assumptions depending on the cavitation phenomenon as mentioned in Introduction. The enzyme inactivation by US is mainly attributed to the mechanical and chemical effects of cavitation (Raviyan et al., 2005). When a high power US wave propagates in a liquid medium, cavitation bubbles are generated due to rapid change in pressure. These micro bubbles collapse violently in the succeeding compression cycles of a propagated sonic wave. The collapse of these micro bubbles generates very high-localized temperature and pressure (Sala et al., 1995). Moreover, US can also create shock waves during the cavitation and the micro bubbles in the liquid are changed shape and size continuously. This changing actions cause an acoustic stream adjacent and severe shear stresses are observed. The stresses generally result in enzyme denaturation, because the secondary and tertiary structures of enzyme are altered resulting into loss in their biological activity (Tian et al., 2004). In addition, sonication also has chemical effects involving H● and OH● free radicals (López et al., 1994). These free radicals could be removed some unwanted amino acid residues which are used for structure stability, substrate binding or catalytic functions (Kuldiloke, 2002). These two mechanisms are responsible for the synergistic effect of MTS enzyme inactivation process.

Table 3.18. Relationship between temperature and experimental and theoretical D_{MTS} values (calculated with Eq.2. 9) of PPO and PME in apple and orange juices and the synergistic effect of the combined MTS treatment at different temperatures (calculated with Eq. 2.10).

Enzymes	Treatment media	pH	Pressure (kPa)	Temperature (˚C)	D_{MS} (min)	D_{Heat} (min)	Theoretical D_{MTS} (min)	Experimental D_{MTS} (min)	% synergism
PPO	Apple Juice	3.4	200	35	8.46	-	-	-	-
				50	-	24.64	25.19	4.20	83.33
				60	-	2.20	6.98	0.92	86.83
PME	Orange Juice	3.7	200	35	12.98	-	-	-	-
				50	-	48.96	41.04	13.683	66.67
				60	-	36.83	38.39	4.067	89.40

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

This study was conducted in two parts. In the first part, the inactivation of *Listeria monocytogenes* STCC 5672 and *Escherichia coli* O157:H7 suspended in UHT apple and orange juices by ultrasonic waves under pressure at nonlethal (MS) and lethal temperatures (MTS) was evaluated.

The relatively most resistance strains of *L. monocytogenes* and *E. coli* were determined by MS (35 °C, 110 μm, 200 kPa) in the McIlvaine citrate-phosphate buffer at pH 3.5. As a reference, TT (60°C, 200 kPa) was applied in McIlvaine citrate-phosphate buffer at pH 3.5. Significant differences($p \leq 0.05$) were found among the MS resistance of five strains of *L. monocytogenes* and three strains of *E. coli* in pH 3.5 buffer, being *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 the most resistant strains chosen to carry out the evaluation of the effect of the process parameters. Regarding the interspecific differences, *L. monocytogenes* showed higher MS resistance than *E. coli* ($p \leq 0.05$).

The MS resistance of *L. monocytogenes* and *E. coli* was not affected by the pH values studied regardless of the food matrix, buffer *vs.* juices and was similar when suspended in apple juice (pH 3.4), orange juice (pH 3.7) and McIlvaine buffer at a similar pH (3.5) ($p > 0.05$).

The energy transmitted to the fruit juices by ultrasonic waves at different combinations of amplitudes (46.5, 90, 110, and 130.5 μm) and pressures (0, 100, and 200 kPa) was also studied, obtaining an exponential relationship between D_{MS} values and power input: an increase of 116 W increased the inactivation rate approximately 10-fold in both juices. No significant differences ($p > 0.05$) were found among the slopes of the regression lines for the two species studied in both fruit juices, and consequently, between the z_{MS} values, obtaining a z_{MS} mean value of 116 W.

Both increasing the amplitude and pressure caused an increase in the lethality of MS treatment in both fruit juices. Among the selected parameters, it was found that the effective MS microbial inactivation combination was determined as 130.5 μm amplitude and 200 kPa pressure at 35°C. No statistically significant differences ($p > 0.05$) were found among the slopes of the regression lines for both species in both juices, indicating that the magnitude of the effect of ultrasonic wave amplitude on the microbial resistance is similar for *L. monocytogenes* and *E. coli* in both fruit juices. Similar to amplitude parameter, although the significant differences ($p \leq 0.05$) were found between the D_{MS} values and the magnitude of pressure, no statistically significant differences ($p > 0.05$) were found among the slopes of the regression lines for both species in both juices. Thus,

it was concluded that the effect of the amplitude and pressure on MS resistance would be the same regardless of the treatment media and the species investigated.

Regarding the kinetics of inactivation, while survival curves obtained after MS/MTS treatment were linear ($R^2 \geq 0.97$), those obtained after TT showed a downward concavity. The concave downward profiles only appeared when apple and orange juices were used as treatment media, but not when buffer of the same pH was used as treatment media. The lethality of TT was increased with temperature for both species in both juices. These deviations from linearity disappeared by applying ultrasound; both simplifying the kinetics of inactivation and enabling the calculation of the treatment time required for a specific level of inactivation. On the other hand, in contrast to the interspecific strain behavior shown under MS treatments, it should be noticed that the Gram-negative *E. coli* O157:H7 displayed a higher heat resistant than the Gram-positive *L. monocytogenes* STCC 5672 in both fruit juices. These results would point out *E. coli* O157:H7 instead of *L. monocytogenes* as a target microorganism when processing fruit juices by heat.

The inactivation by MS was independent of temperature up to a threshold temperature above, which the rate of inactivation quickly increased with temperature. This limiting temperature distinguishes between MS and MTS treatments and it is dependent on the microorganism and the treatment media. Among the selected parameters, the best treatment combinations were determined as 110 μm amplitude-200 kPa pressure at 60°C for both species regardless of the treatment media. Despite *L. monocytogenes* STCC 5672 which should be considered as the target microorganism under MS treatments and the same for *E. coli* O157:H7 under heat treatments, no significant differences were found between the D_{MTS} values for both strains at 60 ºC in both juices ($p>0.05$). The comparison of the inactivation by heat with the inactivation by ultrasound treatments shows that both the MS and the combined process (MTS) were more efficient on reducing the microbial population than heat acting alone. MTS showed a synergistic lethal effect in acidic juices, whose magnitude was dependent on the treatment conditions. The maximum synergism was observed when inactivating *L. monocytogenes* in apple juice by MTS (110 μm, 200 kPa) at 60ºC.

In the second part of this study, the inactivation of PPO and PME in freshly squeezed apple and orange juices by ultrasonic waves under pressure at nonlethal (MS) and lethal temperatures (MTS) was evaluated.

The temperature profile of enzyme inactivation in the fruit juices by ultrasonic waves at different combinations of amplitudes (90, 110, 130.5 μm) and pressures (0, 100, 200 kPa) was also studied. The higher temperatures were recorded for the highest ultrasound amplitude (130.5 μm) and pressure (200 kPa), which were 48 °C for apple juice and 52 °C for orange juice, respectively. Amplitude and pressure have significant effect ($p\leq0.05$) on the final temperatures of acidic fruit juices.

The relationship between the inactivation rates of PPO and PME and the energy transmitted by the ultrasound treated freshly squeezed apple and orange juices was

studied at different combination of amplitudes and pressures. An exponential relationship between D_{MS} values and power input was obtained and an increase of 113 W in the energy transferred into the freshly squeezed apple and orange juices by US will make the inactivation rate of PPO and PME increase by 10 times in both juices. No significant differences ($p>0.05$) were found among the slopes of the regression lines for the two natural enzymes studied in both fruit juices, and consequently, between the z_{MS} values, obtaining a z_{MS} mean value of 113 W.

Both increasing the amplitude and the pressure caused an increase in the lethality of MS treatment in both freshly squeezed fruit juices. Among the selected parameters, the effective MS enzyme inactivation combination was determined as 130.5 μm amplitude and 200 kPa pressure at 35°C. No statistically significant differences ($p>0.05$) were found among the slopes of the regression lines of for PPO and PME in apple and orange juices, respectively. Similar to amplitude parameter, although the significant differences ($p\leq0.05$) were found between the D_{MS} values and the magnitude of pressure, no statistically significant differences ($p>0.05$) were found among the slopes of the regression lines of for both enzymes.

During the TT, the inactivation rate of enzymes was positively affected by treatment temperature. The increase in the temperature decreased the D_{TT} values of PPO and PME. Because it has generally been associated with the presence of iso-enzymes of different thermal sensitivity, PME has higher thermo stability than PPO. When the temperature was increased from 25 to 60 °C(for PPO) or to 70°C (for PME) in the TT, PPO enzyme lost almost 98 % of initial activity but PME activity declined slowly and it still needed time to achieve 90 % thermal inactivation.

The MS resistance of both species decreased when heat was applied jointly with ultrasound (MTS), which was more effective on inactivating PPO and PME than the sum of MS and heat acting simultaneously but independently. The comparison of the inactivation by TT with the inactivation by MS treatment shows that the combined process (MTS) was more efficient on reducing the enzyme activity than TT acting alone. MS treatment was less effective on the inactivation of PPO enzyme than heat and MTS. Because PPO is considered as a heat labile enzyme, heat inactivation was more effective on the inactivation of PPO in apple juice after 2 min treatment during MTS; contrary to MS and MTS process, which were more effective on the inactivation of PME in orange juice than on the inactivation of heat, because of the high thermo-stability of PME. According to this study, the ultrasonic resistance of PME in orange juice was much higher than PPO in apple juice. According to treatment conditions, MTS treatment showed a synergistic lethal effect in acidic juices. The magnitude of synergistic effect has almost same values for both enzymes. The maximum synergism was observed when inactivating PME in orange juice by MTS (110 μm, 200 kPa) at 60ºC.

The facts of first part are that (i) pH is a factor of minor relevance in this kind of process as well as the interspecific differences in resistance become smaller, (ii) the bacterial cell inactivation rates of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7 by MS increased

with increase in ultrasonic wave amplitude and pressure, (iii) MTS treatments at a specific temperature are more effective on inactivating microbial loads than TS, MS, or heat, and (iv) the combination of ultrasound and heat treatment (110 µm, 200 kPa, 60ºC) has been demonstrated to be synergistic for the inactivation of *L. monocytogenes* STCC 5672 and *E. coli* O157:H7, are of great advantage for the processing of acidic fruit juices by MTS.

The facts of second part are that (i) amplitude and pressure have significant effect ($p \leq 0.05$) on the final temperatures of acidic fruit juices, (ii) the enzyme inactivation rates of PPO in apple juice and PME in orange juice by MS increased with increase in ultrasonic wave amplitude and pressure, (iii) MTS treatments at a specific temperature are more effective on inactivating natural enzymes of acidic fruit juices than TS, MS, or TT, (iv) MS treatment is less effective on inactivation of PPO than TT, and (v) the combination of ultrasound and heat treatment (110 µm, 200 kPa, 60ºC) has been demonstrated to be synergistic for the inactivation of PPO in apple juice and PME in orange juice, are of great advantage for the processing of acidic fruit juices by MTS.

Overall, the data presented in this study suggest that MTS could be a plausible alternative to current pasteurization treatments for fruit juices. The optimum conditions for treatment will mainly rely on the thermo-tolerance of the target microorganism and target natural enzyme and would correspond to those conditions in which the synergism between the hurdles (ultrasound, pressure, and heat) is maximum.

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APPENDIX A

RESULTS AND DISCUSSION

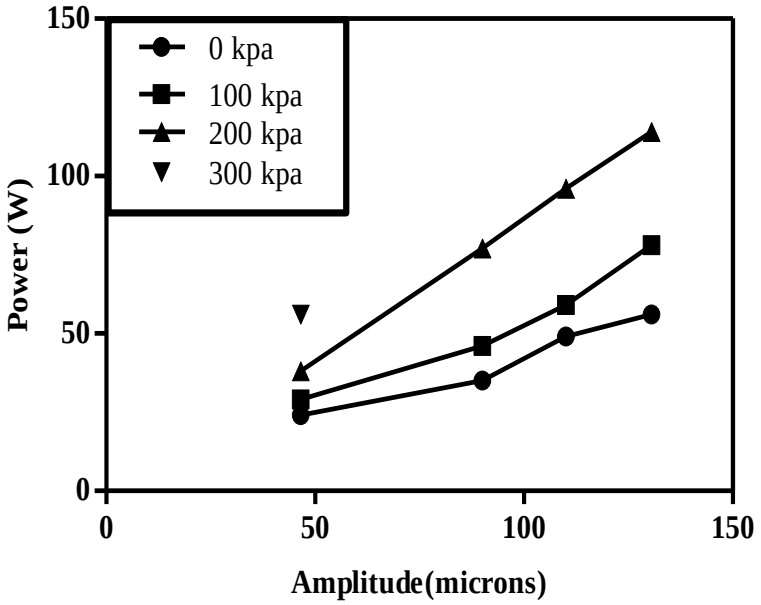

Figure A.1. Pressure effect on the power (W) at different amplitudes for apple juice

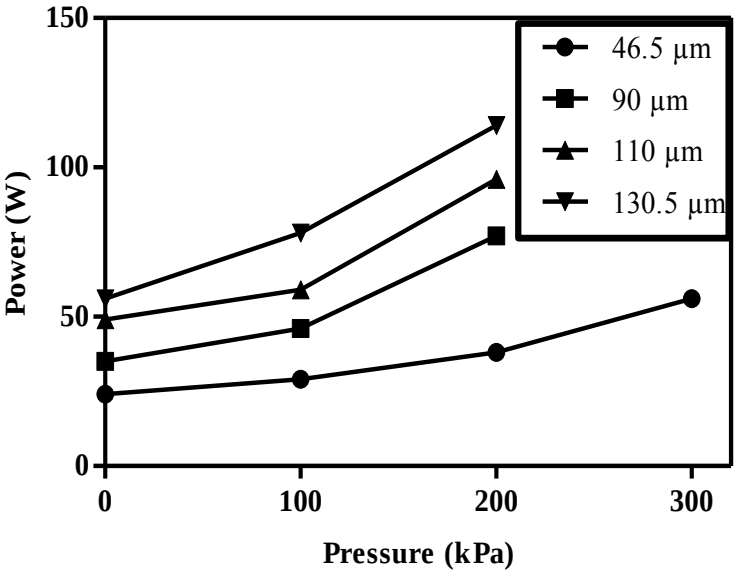

Figure A.2. Amplitude effect on the power (W) at different pressures for apple juice

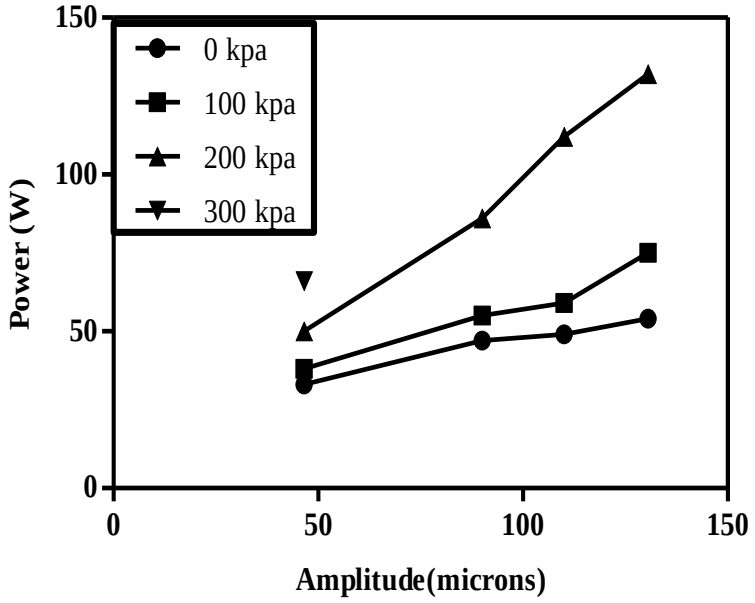

Figure A.3. Pressure effect on the power (W) at different amplitudes for orange juice

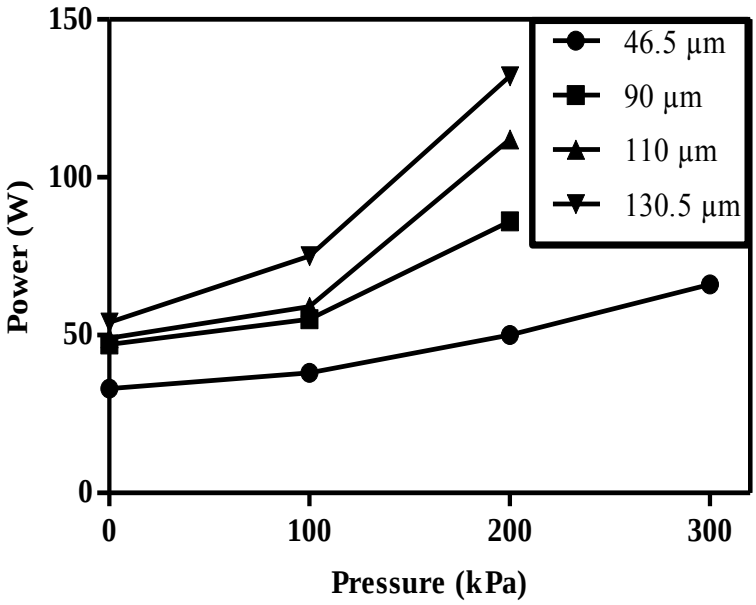

Figure A.4. Amplitude effect on the power (W) at different pressures for orange juice

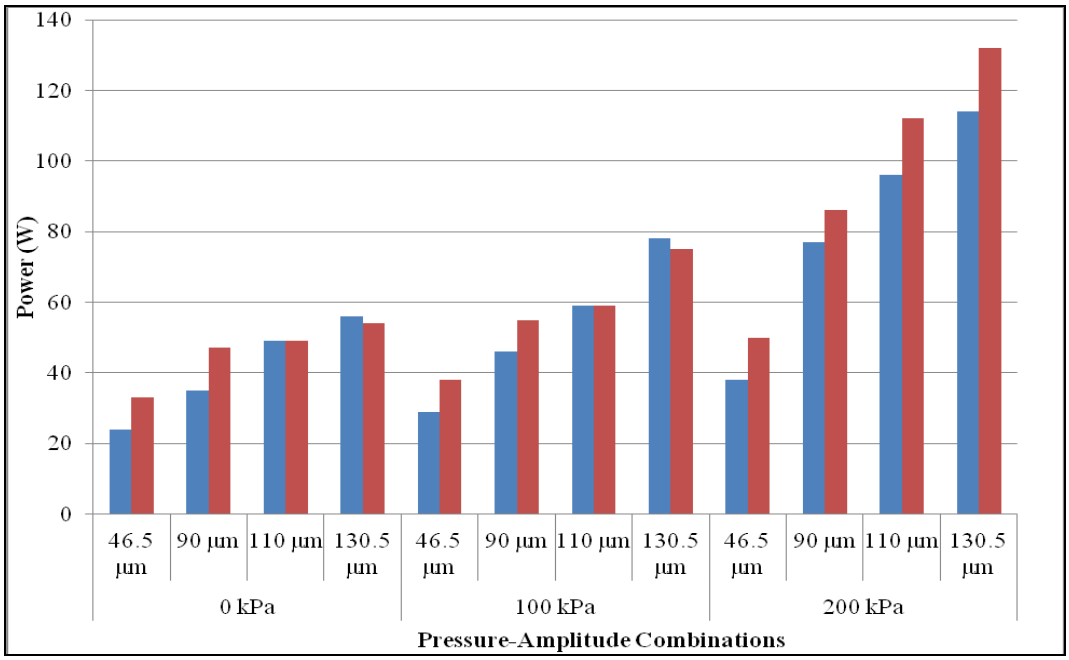

Figure A.5. The power (W) of ultrasound at different amplitude-pressure combinations for apple (blue bar) and orange (red bar) juices

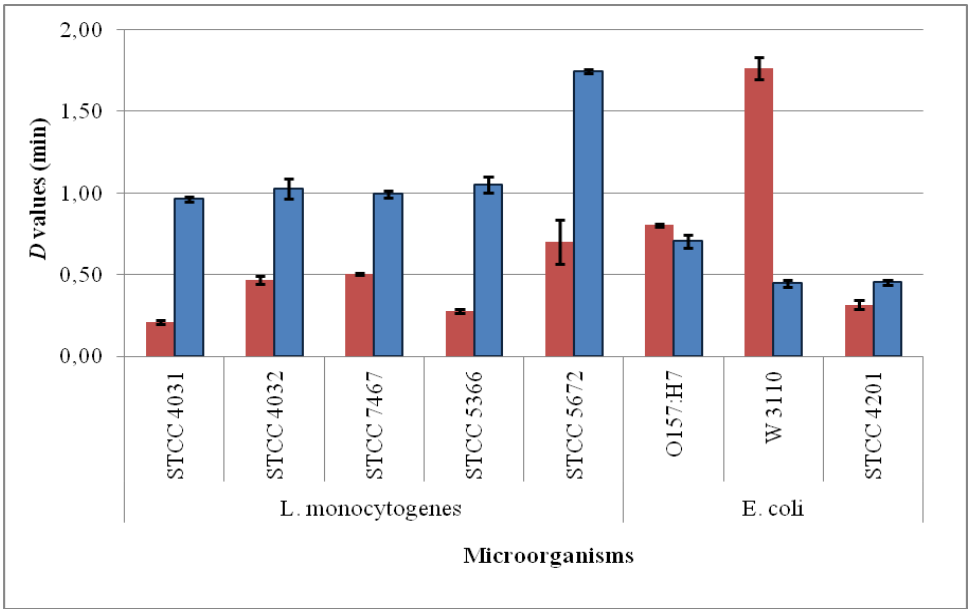

Figure A.6. Resistance parameters [D_{MS} (red bar) and D_{TT} (blue bar) values] of *L. monocytogenes* and *E. coli* to MS (200 kPa, 110 µm, 35 °C) and TT (200 kPa, 60 °C) in McIlvaine citrate-phosphate buffer of pH 3.5.

Table A1. Resistance parameters ($D_{MS/MTS}$) of *Listeria monocytogenes* to ultrasound treatments. ANOVA test ($p \leq 0.05$) was conducted within each group, as shown in Significant level column. To identify intergroup differences after significant differences in the ANOVA test, multiple comparisons were performed using Tukey's test. Values with the same superscript did not show statistically significant differences ($p > 0.05$).

Strain	Treatment conditions					Fit parameters			
	pH	Treatment media	Amplitude (μm)	Pressure (kPa)	T (˚C)	$D_{MS/MTS}$ (min)	*SD*	Significant level	R^2
STCC 4031	3.5	Buffer	110	200	35	0.96^{a}	0.02		0.99
STCC 7467	3.5	Buffer	110	200	35	1.05^{a}	0.05	$p \leq 0.05$	0.99
STCC 5672	3.5	Buffer	110	200	35	1.74^{b}	0.15		0.97
	3.5	Buffer	110	200	35	1.74	0.15		0.97
	7.0	Buffer	110	200	35	1.75	0.04	Ns	0.98
	3.4	Apple Juice	110	200	35	1.81	0.08		0.98
	3.7	Orange Juice	110	200	35	1.87	0.08		0.99
	3.4	Apple Juice	46.5	200	35	3.51^{d}	0.23	$p \leq 0.05$	0.99
	3.4		90	200	35	2.19^{c}	0.08		0.98
	3.4		110	200	35	1.81^{b}	0.08		0.98
	3.4		130.5	200	35	1.32^{a}	0.05		0.99
	3.7	Orange Juice	46.5	200	35	4.41^{c}	0.71	$p \leq 0.05$	0.99
	3.7		90	200	35	3.40^{b}	0.16		0.99
	3.7		110	200	35	1.87^{a}	0.08		0.99
	3.7		130.5	200	35	1.05^{a}	0.04		0.99
STCC 5672	3.4	Apple Juice	110	0	35	5.83^{b}	0.48	$p \leq 0.05$	0.98
	3.4		110	100	35	2.51^{a}	0.08		0.99
	3.4		110	200	35	1.81^{a}	0.08		0.98
	3.7	Orange Juice	110	0	35	4.25^{c}	0.03	$p \leq 0.05$	0.98
	3.7		110	100	35	3.05^{b}	0.18		0.99
	3.7		110	200	35	1.87^{a}	0.08		0.99
	3.4	Apple Juice	110	200	35	1.81^{d}	0.08	$p \leq 0.05$	0.98
	3.4		110	200	50	1.05^{c}	0.00		0.99
	3.4		110	200	55	0.77^{b}	0.05		0.97
	3.4		110	200	60	0.23^{a}	0.01		0.97
	3.7	Orange Juice	110	200	35	1.87^{d}	0.08	$p \leq 0.05$	0.99
	3.7		110	200	50	1.20^{c}	0.00		0.99
	3.7		110	200	55	0.76^{b}	0.12		0.96
	3.7		110	200	60	0.31^{a}	0.01		0.95

SD, Standard deviation; Ns, no significant ($p > 0.05$); $R^{2.}$, determination coefficient.

Table A2. Resistance parameters ($D_{MS/MTS}$) of *Escherichia coli* to ultrasound treatments. ANOVA test ($p \leq 0.05$) was conducted within each group, as shown in Significant level column. To identify intergroup differences after significant differences in the ANOVA test, multiple comparisons were performed using Tukey's test. Values with the same superscript did not show statistically significant differences ($p > 0.05$).

Strains	Treatment conditions					Fit parameters			
	pH	Treatment media	Amplitude (μm)	Pressure (kPa)	T (˚C)	$D_{MS/MTS}$ (min)	*SD*	Significant level	R^2
STCC 4201	3.5	Buffer	110	200	35	0.45^{a}	0.02		0.99
W 3110	3.5	Buffer	110	200	35	0.45^{a}	0.01	$p \leq 0.05$	0.99
O157:H7	3.5	Buffer	110	200	35	0.76^{b}	0.04		0.98
O157:H7	3.5	Buffer	110	200	35	0.76	0.04		0.98
	7.0	Buffer	110	200	35	0.80	0.04	Ns	0.95
	3.4	Apple Juice	110	200	35	0.92	0.03		0.98
	3.7	Orange Juice	110	200	35	0.93	0.00		0.99
	3.4	Apple Juice	46.5	200	35	3.59^{b}	0.10		0.96
	3.4		90	200	35	1.09^{a}	0.11	$p \leq 0.05$	0.99
	3.4		110	200	35	0.92^{a}	0.03		0.98
	3.4		130.5	200	35	0.85^{a}	0.12		0.98
	3.7	Orange Juice	46.5	200	35	3.07^{c}	0.06		0.99
	3.7		90	200	35	1.50^{b}	0.04	$p \leq 0.05$	0.99
	3.7		110	200	35	0.93^{a}	0.00		0.99
	3.7		130.5	200	35	0.82^{a}	0.08		0.99
	3.4	Apple Juice	110	0	35	2.41^{c}	0.33		0.99
	3.4		110	100	35	1.46^{b}	0.10	$p \leq 0.05$	0.99
	3.4		110	200	35	0.92^{a}	0.03		0.98
	3.7	Orange Juice	110	0	35	2.79^{c}	0.45		0.98
	3.7		110	100	35	1.71^{b}	0.02	$p \leq 0.05$	0.98
	3.7		110	200	35	0.93^{a}	0.00		0.99
	3.4	Apple Juice	110	200	35	0.92^{c}	0.03		0.98
	3.4		110	200	50	0.89^{c}	0.08	$p \leq 0.05$	0.99
	3.4		110	200	55	0.52^{b}	0.07		0.98
	3.4		110	200	60	0.27^{a}	0.02		0.96
	3.7	Orange Juice	110	200	35	0.93^{d}	0.00		0.99
	3.7		110	200	50	0.77^{c}	0.06	$p \leq 0.05$	0.99
	3.7		110	200	55	0.59^{b}	0.01		0.99
	3.7		110	200	60	0.29^{a}	0.01		0.92

SD, Standard deviation; Ns, no significant ($p > 0.05$); R^2, determination coefficient.

APPENDIX B

STATISTICAL ANALYSIS OF MICROBIAL INACTIVATION BY US

Table B1. ANOVA table for the lethal effect of MS inactivation on five strains of *L. monocytogenes* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{MS} values).

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	1,773(a)	4	,443	188,849	,000
Intercept	20,859	1	20,859	8884,415	,000
Strains	1,773	4	,443	188,849	,000
Error	,023	10	,002		
Total	22,656	15			
Corrected Total	1,797	14			

a R Squared = ,987 (Adjusted R Squared = ,982)

Table B2. Tukey test table for the lethal effect of MS inactivation on five strains of *L. monocytogenes* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{MS} values).

Strains	N	Subset	
	1	2	1
L. monocytogenes STCC 4031	3	,9622	
L. monocytogenes STCC 7467	3	,9933	
L. monocytogenes STCC 4032	3	1,0256	
L. monocytogenes STCC 5366	3	1,0507	
L. monocytogenes STCC 5672	3		1,8643
Sig.		,241	1,000

Table B3. ANOVA table for the lethal effect of TT inactivation on five strains of *L. monocytogenes* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{TT} values).

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	,457(a)	4	,114	28,725	,000
Intercept	2,804	1	2,804	705,412	,000
Strains	,457	4	,114	28,725	,000
Error	,040	10	,004		
Total	3,300	15			
Corrected Total	,496	14			

Table B4. Tukey test table for the lethal effect of TT inactivation on five strains of *L. monocytogenes* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{TT} values).

Strains	N	Subset		
	1	2	3	1
L. monocytogenes STCC 4031	3	,2106		
L. monocytogenes STCC 5366	3	,2766		
L. monocytogenes STCC 4032	3		,4686	
L. monocytogenes STCC 7467	3		,5046	
L. monocytogenes STCC 5672	3			,7012
Sig.		,707	,952	1,000

Table B5. ANOVA table for the lethal effect of MS inactivation on three strains of *E. coli* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{MS} values).

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	,131(a)	2	,065	93,212	,000
Intercept	2,572	1	2,572	3668,911	,000
Strains	,131	2	,065	93,212	,000
Error	,004	6	,001		
Total	2,707	9			
Corrected Total	,135	8			

a R Squared = ,969 (Adjusted R Squared = ,958)

Table B6. Tukey test table for the lethal effect of MS inactivation on three strains of *E. coli* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{MS} values).

Strains	N	Subset	
	1	2	1
E. coli W3110	3	,4452	
E. coli STCC 4201	3	,4537	
E. coli O157:H7	3		,7050
Sig.		,920	1,000

Table B7. ANOVA table for the lethal effect of TT inactivation on three strains of *E. coli* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{TT} values).

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	3,244(a)	2	1,622	888,830	,000
Intercept	8,296	1	8,296	4546,815	,000
Strains	3,244	2	1,622	888,830	,000
Error	,011	6	,002		
Total	11,551	9			
Corrected Total	3,254	8			

a R Squared = ,997 (Adjusted R Squared = ,996)

Table B8. Tukey test table for the lethal effect of TT inactivation on three strains of *E. coli* in McIlvaine citrate-phosphate buffer of pH 3.5
(Dependent Variable: D_{TT} values).

Strains	N	Subset		
	1	2	3	1
E. coli STCC 4201	3	,3164		
E. coli O157:H7	3		,8026	
E. coli W3110	3			1,7613
Sig.		1,000	1,000	1,000

Table B9. ANOVA table for the lethal effect of MS inactivation on *L. monocytogenes* STCC 5672 in four different treatment media at different pH
(Dependent variable: Log D_{MS} values)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	,037(a)	3	,012	1,372	,319
Intercept	38,557	1	38,557	4316,166	,000
medium	,037	3	,012	1,372	,319
Error	,071	8	,009		
Total	38,665	12			
Corrected Total	,108	11			

a R Squared = ,340 (Adjusted R Squared = ,092)

Table B10. Tukey test table for the lethal effect of MS inactivation on *L. monocytogenes* STCC 5672 in four different treatment media at different pH
(Dependent variable: Log D_{MS} values)

Medium	N	Subset
	1	1
McIlavine buffer (pH 7.0)	3	1,7373
McIlavine buffer (pH 3.5)	3	1,7457
Apple juice (pH 3.4)	3	1,8133
Orange juice (pH 3.7)	3	1,8737
Sig.		,354

Table B11. ANOVA table for the lethal effect of MS inactivation on *E. coli* O157:H7 in four different treatment media at different pH
(Dependent variable: Log D_{MS} values)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	,066(a)	3	,022	5,470	,024
Intercept	8,681	1	8,681	2174,825	,000
medium	,066	3	,022	5,470	,024
Error	,032	8	,004		
Total	8,778	12			
Corrected Total	,097	11			

a R Squared = ,672 (Adjusted R Squared = ,549)

Table B12. Tukey test table for the lethal effect of MS inactivation on *E. coli* O157:H7 in four different treatment media at different pH
(Dependent variable: Log D_{MS} values)

Medium	N	Subset	
	1	2	1
McIlavine buffer (pH 3.5)	3	,757067	
McIlvaine buffer (pH 7.0)	3	,799533	,799533
Apple juice (pH 3.4)	3	,917367	,917367
Orange juice (pH 3.7)	3		,928200
Sig.		,057	,135

Table B13. ANOVA table for effect of ultrasonic wave amplitude on the MS resistance of *L. monocytogenes* STCC 5672 in apple juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	312,940(a)	11	28,449	424,550	,000
Intercept	689,378	1	689,378	10287,677	,000
amplitude	127,598	3	42,533	634,720	,000
pressure	161,530	2	80,765	1205,269	,000
amplitude * pressure	23,812	6	3,969	59,226	,000
Error	1,608	24	,067		
Total	1003,926	36			
Corrected Total	314,549	35			

a R Squared = ,995 (Adjusted R Squared = ,993)

Table B14. Tukey test table for effect of ultrasonic wave amplitude on the MS resistance of *L. monocytogenes* STCC 5672 in apple juice
(Dependent variables: Log D_{MS} values)

Amplitude	N	Subset			
	1	2	3	4	1
90% or 130,5 µm	9	2,5732			
75% or 110 µm	9		3,3922		
60% or 90 µm	9			4,0261	
30% or 46,5 µm	9				7,5124
Sig.		1,000	1,000	1,000	1,000

Table B15. ANOVA table for effect of ultrasonic wave amplitude on the MS resistance of *E. coli* O157:H7 in apple juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	104,629(a)	11	9,512	211,326	,000
Intercept	224,063	1	224,063	4978,087	,000
Amplitude	73,241	3	24,414	542,404	,000
Pressure	26,118	2	13,059	290,142	,000
amplitude * pressure	5,270	6	,878	19,516	,000
Error	1,080	24	,045		
Total	329,773	36			
Corrected Total	105,710	35			

a R Squared = ,990 (Adjusted R Squared = ,985)

Table B16. Tukey test table for effect of ultrasonic wave amplitude on the MS resistance of *E. coli* O157:H7 in apple juice
(Dependent variables: Log D_{MS} values)

Amplitude	N	Subset		
	1	2	3	1
90% or 130.5 μm	9	1.4540		
75% or 110 μm	9	1.6115		
60% or 90 μm	9		1.9697	
30% or 46.5 μm	9			4.9440
Sig.		.412	1.000	1.000

Table B17. ANOVA table for effect of ultrasonic wave amplitude on the MS resistance of *L. monocytogenes* STCC 5672 in orange juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	252,250(a)	11	22,932	312,015	,000
Intercept	670,370	1	670,370	9121,198	,000
amplitude	130,169	3	43,390	590,370	,000
pressure	99,839	2	49,919	679,212	,000
amplitude * pressure	22,242	6	3,707	50,439	,000
Error	1,764	24	,073		
Total	924,384	36			
Corrected Total	254,014	35			

a R Squared = ,993 (Adjusted R Squared = ,990)

Table B18. Tukey test table for effect of ultrasonic wave amplitude on the MS resistance of *L. monocytogenes* STCC 5672 in orange juice
(Dependent variables: Log D_{MS} values)

amplitude	N	Subset			
	1	2	3	4	1
90% or 130,5 µm	9	2,3181			
75% or 110 µm	9		3,0562		
60% or 90 µm	9			4,6039	
30% or 46,5 µm	9				7,2828
Sig.		1,000	1,000	1,000	1,000

Table B19. ANOVA table for effect of ultrasonic wave amplitude on the MS resistance of *E. coli* O157:H7 in orange juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	120,806(a)	11	10,982	477,240	,000
Intercept	285,355	1	285,355	12400,111	,000
Amplitude	62,173	3	20,724	900,577	,000
Pressure	51,870	2	25,935	1127,004	,000
amplitude * pressure	6,763	6	1,127	48,983	,000
Error	,552	24	,023		
Total	406,714	36			
Corrected Total	121,359	35			

a R Squared = ,995 (Adjusted R Squared = ,993)

Table B20. Tukey test table for effect of ultrasonic wave amplitude on the MS resistance of *E. coli* O157:H7 in orange juice
(Dependent variables: Log D_{MS} values)

Amplitude	N	Subset			
	1	2	3	4	1
90% or 130,5 µm	9	1,5857			
75% or 110 µm	9		1,9198		
60% or 90 µm	9			2,7974	
30% or 46,5 µm	9				4,9587
Sig.		1,000	1,000	1,000	1,000

Table B21. ANOVA table for slopes of amplitude linear equation of the MS resistance of *L. monocytogenes* STCC 5672 in apple and orange juices
(Dependent variables: Amplitude linear equation slopes)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	3,47E-006(a)	2	1,73E-006	1,980	,283
Intercept	,000	1	,000	247,801	,001
Pressure	3,47E-006	2	1,73E-006	1,980	,283
Error	2,63E-006	3	8,76E-007		
Total	,000	6			
Corrected Total	6,10E-006	5			

a R Squared = ,569 (Adjusted R Squared = ,282)

Table B22. Tukey test table for slopes of amplitude linear equation of the MS resistance of *L. monocytogenes* STCC 5672 in apple and orange juices
(Dependent variables: Amplitude linear equation slopes)

pressure	N	Subset
	1	1
100 kPa	2	-,00690250
200 kPa	2	-,00610050
0 kPa	2	-,00504550
Sig.		,263

Table B23. ANOVA table for slopes of amplitude linear equation of the MS resistance of *E. coli* O157:H7 in apple and orange juices
(Dependent variables: Amplitude linear equation slopes)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	2,24E-006(a)	2	1,12E-006	13,726	,031
Intercept	,000	1	,000	3139,828	,000
Pressure	2,24E-006	2	1,12E-006	13,726	,031
Error	2,45E-007	3	8,17E-008		
Total	,000	6			
Corrected Total	2,49E-006	5			

a R Squared = ,901 (Adjusted R Squared = ,836)

Table B24. Tukey test table for slopes of amplitude linear equation of the MS resistance of *E. coli* O157:H7 in apple and orange juices
(Dependent variables: Amplitude linear equation slopes)

Pressure	N	Subset	
	1	2	1
200 kPa	2	-,00740050	
100 kPa	2		-,00617050
0 kPa	2		-,00604550
Sig.		1,000	,903

Table B25. ANOVA table for effect of pressure on the MS resistance of *L. monocytogenes* STCC 5672 in apple juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	312,940(a)	11	28,449	424,550	,000
Intercept	689,378	1	689,378	10287,677	,000
Amplitude	127,598	3	42,533	634,720	,000
Pressure	161,530	2	80,765	1205,269	,000
amplitude * pressure	23,812	6	3,969	59,226	,000
Error	1,608	24	,067		
Total	1003,926	36			
Corrected Total	314,549	35			

a R Squared = ,995 (Adjusted R Squared = ,993)

Table B26. Tukey test table for effect of pressure on the MS resistance of *L. monocytogenes* STCC 5672 in apple juice
(Dependent variables: Log D_{MS} values)

pressure	N	Subset		
	1	2	3	1
200 kPa	12	2,2093		
100 kPa	12		3,6679	
0 kPa	12			7,2508
Sig.		1,000	1,000	1,000

Table B27. ANOVA table for effect of pressure on the MS resistance of *L. monocytogenes* STCC 5672 in orange juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	252,250(a)	11	22,932	312,015	,000
Intercept	670,370	1	670,370	9121,198	,000
amplitude	130,169	3	43,390	590,370	,000
Pressure	99,839	2	49,919	679,212	,000
amplitude * pressure	22,242	6	3,707	50,439	,000
Error	1,764	24	,073		
Total	924,384	36			
Corrected Total	254,014	35			

a R Squared = ,993 (Adjusted R Squared = ,990)

Table B28. Tukey test table for effect of pressure on the MS resistance of *L. monocytogenes* STCC 5672 in orange juice
(Dependent variables: Log D_{MS} values)

pressure	N	Subset		
	1	2	3	1
200 kPa	12	2,6835		
100 kPa	12		3,6604	
0 kPa	12			6,6018
Sig.		1,000	1,000	1,000

Table B29. ANOVA table for effect of pressure on the MS resistance of *E. coli* O157:H7 in apple juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	104,629(a)	11	9,512	211,326	,000
Intercept	224,063	1	224,063	4978,087	,000
amplitude	73,241	3	24,414	542,404	,000
pressure	26,118	2	13,059	290,142	,000
amplitude * pressure	5,270	6	,878	19,516	,000
Error	1,080	24	,045		
Total	329,773	36			
Corrected Total	105,710	35			

a R Squared = ,990 (Adjusted R Squared = ,985)

Table B30. Tukey test table for effect of pressure on the MS resistance of *E. coli* O157:H7 in apple juice
(Dependent variables: Log D_{MS} values)

pressure	N	Subset		
	1	2	3	1
200 kPa	12	1,6104		
100 kPa	12		2,2287	
0 kPa	12			3,6453
Sig.		1,000	1,000	1,000

Table B31. ANOVA table for effect of pressure on the MS resistance of *E. coli* O157:H7 in orange juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	120,806(a)	11	10,982	477,240	,000
Intercept	285,355	1	285,355	12400,111	,000
Amplitude	62,173	3	20,724	900,577	,000
Pressure	51,870	2	25,935	1127,004	,000
amplitude * pressure	6,763	6	1,127	48,983	,000
Error	,552	24	,023		
Total	406,714	36			
Corrected Total	121,359	35			

a R Squared = ,995 (Adjusted R Squared = ,993)

Table B32. Tukey test table for effect of pressure on the MS resistance of *E. coli* O157:H7 in orange juice
(Dependent variables: Log D_{MS} values)

pressure	N	Subset		
	1	2	3	1
200 kPa	12	1,5813		
100 kPa	12		2,4230	
0 kPa	12			4,4419
Sig.		1,000	1,000	1,000

Table B33. ANOVA table for slopes of pressure linear equation of the MS resistance of *L. monocytogenes* STCC 5672 in apple and orange juices
(Dependent variables: Pressure linear equation slopes)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	1,10E-006(a)	3	3,67E-007	1,547	,333
Intercept	4,93E-005	1	4,93E-005	207,653	,000
Amplitude	1,10E-006	3	3,67E-007	1,547	,333
Error	9,49E-007	4	2,37E-007		
Total	5,13E-005	8			
Corrected Total	2,05E-006	7			

a R Squared = ,537 (Adjusted R Squared = ,190)

Table B34. Tukey test table for slopes of pressure linear equation of the MS resistance of *L. monocytogenes* STCC 5672 in apple and orange juices
(Dependent variables: Pressure linear equation slopes)

amplitude	N	Subset
	1	1
130.5 µm	2	-,00307500
110 µm	2	-,00247500
46.5 µm	2	-,00230000
90 µm	2	-,00207500
Sig.		,306

Table B35. ANOVA table for slopes of pressure linear equation of the MS resistance of *E. coli* O157:H7 in apple and orange juices
(Dependent variables: Pressure linear equation slopes)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6,93E-007(a)	3	2,31E-007	1,591	,324
Intercept	3,81E-005	1	3,81E-005	261,938	,000
amplitude	6,93E-007	3	2,31E-007	1,591	,324
Error	5,81E-007	4	1,45E-007		
Total	3,93E-005	8			
Corrected Total	1,27E-006	7			

a R Squared = ,544 (Adjusted R Squared = ,202)

Table B36. Tukey test table for slopes of pressure linear equation of the MS resistance of *E. coli* O157:H7 in apple and orange juices
(Dependent variables: Pressure linear equation slopes)

amplitude	N	Subset
	1	1
110 µm	2	-,00250000
90 µm	2	-,00227500
130.5 µm	2	-,00225000
46.5 µm	2	-,00170000
Sig.		,293

Table B37. ANOVA table for the lethal effect of TT inactivation on *L. monocytogenes* STCC 5672 in apple and orange juices
(Dependent Variable: D_{TT} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3,649(a)	5	,730	475,995	,000
Intercept	1,614	1	1,614	1052,612	,000
Temperature	3,639	2	1,819	1186,482	,000
Medium	,000	1	,000	,293	,598
temperature * medium	,010	2	,005	3,359	,069
Error	,018	12	,002		
Total	5,282	18			
Corrected Total	3,668	17			

a R Squared = ,995 (Adjusted R Squared = ,993)

Table B38. Tukey test table for the lethal effect of TT inactivation on *L. monocytogenes* STCC 5672 in apple and orange juices.
(Dependent Variable: D_{TT} values).

temperature	N	Subset		
	1	2	3	1
60 °C	6	-,2883		
55 °C	6		,3833	
50 °C	6			,8033
Sig.		1,000	1,000	1,000

Table B39. ANOVA table for the lethal effect of TT inactivation on *E.coli* O157:H7 in apple and orange juices
(Dependent Variable: D_{TT} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	8,569(a)	5	1,714	1504,749	,000
Intercept	11,777	1	11,777	10341,151	,000
temperature	8,522	2	4,261	3741,546	,000
Medium	,023	1	,023	19,980	,001
temperature * medium	,024	2	,012	10,337	,002
Error	,014	12	,001		
Total	20,360	18			
Corrected Total	8,582	17			

a R Squared = ,998 (Adjusted R Squared = ,998)

Table B40. Tukey test table for the lethal effect of TT inactivation on *E.coli* O157:H7 in apple and orange juices.
(Dependent Variable: D_{TT} values).

temperature	N	Subset		
	1	2	3	1
60 °C	6	-,0750		
55 °C	6		,8983	
50 °C	6			1,6033
Sig.		1,000	1,000	1,000

Table B41. ANOVA table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in apple juice at 100 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,526(a)	2	,263	877,370	,000
Intercept	,082	1	,082	273,926	,000
Pressure	,000	0	.	.	.
Temperature	,526	2	,263	877,370	,000
pressure * temperature	,000	0	.	.	.
Error	,002	6	,000		
Total	,610	9			
Corrected Total	,528	8			

a R Squared = ,997 (Adjusted R Squared = ,995)

Table B42. Tukey test table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in apple juice at 100 kPa constant pressure.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset	
	1	2	1
60 °C	3	-,4367	
55 °C	3		,0533
50 °C	3		,0967
Sig.		1,000	,050

Table B43. ANOVA table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in apple juice at 200 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,755(a)	2	,377	849,025	,000
Intercept	,553	1	,553	1243,225	,000
Pressure	,000	0	.	.	.
Temperature	,755	2	,377	849,025	,000
pressure * temperature	,000	0	.	.	.
Error	,003	6	,000		
Total	1,310	9			
Corrected Total	,757	8			

a R Squared = ,996 (Adjusted R Squared = ,995)

Table B44. Tukey test table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in apple juice at 200 kPa constant pressure.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60 °C	3	-,6500		
55 °C	3		-,1133	
50 °C	3			,0200
Sig.		1,000	1,000	1,000

Table B45. ANOVA table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in orange juice at 100 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,629(a)	2	,314	1768,750	,000
Intercept	,171	1	,171	961,000	,000
Pressure	,000	0	.	.	.
Temperature	,629	2	,314	1768,750	,000
pressure * temperature	,000	0	.	.	.
Error	,001	6	,000		
Total	,801	9			
Corrected Total	,630	8			

a R Squared = ,998 (Adjusted R Squared = ,998)

Table B46. Tukey test table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in orange juice at 100 kPa constant pressure.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60 °C	3	-,4933		
55 °C	3		-,0600	
50 °C	3			,1400
Sig.		1,000	1,000	1,000

Table B47. ANOVA table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in orange juice at 200 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,531(a)	2	,266	140,635	,000
Intercept	,306	1	,306	162,094	,000
Pressure	,000	0	.	.	.
Temperature	,531	2	,266	140,635	,000
pressure * temperature	,000	0	.	.	.
Error	,011	6	,002		
Total	,849	9			
Corrected Total	,543	8			

a R Squared = ,979 (Adjusted R Squared = ,972)

Table B48. Tukey test table for the lethal effect of MTS inactivation on *L. monocytogenes* STCC 5672 in orange juice at 200 kPa constant pressure.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60 °C	3	-,5067		
55 °C	3		-,1267	
50 °C	3			,0800
Sig.		1,000	1,000	1,000

Table B49. ANOVA table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in apple juice at 100 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,424(a)	2	,212	264,875	,000
Intercept	,348	1	,348	435,125	,000
Pressure	,000	0	.	.	.
Temperature	,424	2	,212	264,875	,000
pressure * temperature	,000	0	.	.	.
Error	,005	6	,001		
Total	,777	9			
Corrected Total	,429	8			

a R Squared = ,989 (Adjusted R Squared = ,985)

Table B50. Tukey test table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in apple juice at 100 kPa constant pressure.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60 °C	3	-,4500		
55 °C	3		-,2200	
50 °C	3			,0800
Sig.		1,000	1,000	1,000

Table B51. ANOVA table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in apple juice at 200 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,407(a)	2	,203	96,321	,000
Intercept	,822	1	,822	389,389	,000
Pressure	,000	0	.	.	.
Temperature	,407	2	,203	96,321	,000
pressure * temperature	,000	0	.	.	.
Error	,013	6	,002		
Total	1,241	9			
Corrected Total	,419	8			

a R Squared = ,970 (Adjusted R Squared = ,960)

Table B52. Tukey test table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in apple juice at 200 kPa constant pressure.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60 °C	3	-,5700		
55 °C	3		-,2867	
50 °C	3			-,0500
Sig.		1,000	1,000	1,000

Table B53. ANOVA table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in orange juice at 100 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,460(a)	2	,230	481,000	,000
Intercept	,227	1	,227	475,558	,000
Pressure	,000	0	.	.	.
Temperature	,460	2	,230	481,000	,000
pressure * temperature	,000	0	.	.	.
Error	,003	6	,000		
Total	,690	9			
Corrected Total	,462	8			

a R Squared = ,994 (Adjusted R Squared = ,992)

Table B54. Tukey test table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in orange juice at 100 kPa constant pressure.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60 °C	3	-,4700		
55 °C	3		-,0667	
50 °C	3			,0600
Sig.		1,000	1,000	1,000

Table B55. ANOVA table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in orange juice at 200 kPa constant pressure
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	,278(a)	2	,139	266,617	,000
Intercept	,774	1	,774	1482,894	,000
Pressure	,000	0	.	.	.
Temperature	,278	2	,139	266,617	,000
pressure * temperature	,000	0	.	.	.
Error	,003	6	,001		
Total	1,056	9			
Corrected Total	,282	8			

a R Squared = ,989 (Adjusted R Squared = ,985)

Table B56. Tukey test table for the lethal effect of MTS inactivation on *E.coli* O157:H7 in orange juice at 200 kPa constant pressure
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60 °C	3	-,5333		
55 °C	3		-,2300	
50 °C	3			-,1167
Sig.		1,000	1,000	1,000

APPENDIX C

STATISTICAL ANALYSIS OF ENZYME INACTIVATION BY US

Table C1. ANOVA table for effect of ultrasonic wave amplitude on the MS resistance of PPO in apple juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	2121,030(a)	5	424,206	156,125	,000
Intercept	4458,009	1	4458,009	1640,732	,000
Pressure	894,166	1	894,166	329,090	,000
Amplitude	856,874	2	428,437	157,683	,000
Pressure * amplitude	369,991	2	184,996	68,086	,000
Error	32,605	12	2,717		
Total	6611,644	18			
Corrected Total	2153,635	17			

a R Squared = ,985 (Adjusted R Squared = ,979)

Table C2. Tukey test table for effect of ultrasonic wave amplitude on the MS resistance of PPO in apple juice
(Dependent variables: Log D_{MS} values)

Amplitude	N	Subset		
	1	2	3	1
130,5	6	8,0715		
110	6		14,3425	
90	6			24,7983
Sig.		1,000	1,000	1,000

Table C3. ANOVA table for effect of ultrasonic wave amplitude on the MS resistance of PME in orange juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	237,006(a)	2	118,503	5,459	,045
Intercept	1670,602	1	1670,602	76,957	,000
amplitude	237,006	2	118,503	5,459	,045
Error	130,250	6	21,708		
Total	2037,858	9			
Corrected Total	367,256	8			

a R Squared = ,645 (Adjusted R Squared = ,527)

Table C4. Tukey test table for effect of ultrasonic wave amplitude on the MS resistance of PME in orange juice
(Dependent variables: Log D_{MS} values)

	N	Subset	
amplitude	1	2	1
130,5	3	7,6863	
110	3	12,9800	12,9800
90	3		20,2067
Sig.		,402	,219

Table C5. ANOVA table for effect of pressure on the MS resistance of PPO in apple juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	472,232(a)	2	236,116	105,996	,000
Intercept	1529,827	1	1529,827	686,763	,000
Pressure	472,232	2	236,116	105,996	,000
Amplitude	,000	0	.	.	.
Pressure * amplitude	,000	0	.	.	.
Error	13,366	6	2,228		
Total	2015,424	9			
Corrected Total	485,597	8			

a R Squared = ,972 (Adjusted R Squared = ,963)

Table C6. Tukey test table for effect of pressure on the MS resistance of PPO in apple juice
(Dependent variables: Log D_{MS} values)

Pressure	N	Subset		
	1	2	3	1
200	3	5,8997		
100	3		10,2433	
0	3			22,9700
Sig.		1,000	1,000	1,000

Table C7. ANOVA table for effect of pressure on the MS resistance of PME in orange juice
(Dependent variables: Log D_{MS} values)

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	1169,317(a)	2	584,658	51,130	,000
Intercept	4655,788	1	4655,788	407,165	,000
Pressure	1169,317	2	584,658	51,130	,000
Error	68,608	6	11,435		
Total	5893,712	9			
Corrected Total	1237,925	8			

a R Squared = ,945 (Adjusted R Squared = ,926)

Table C8. Tukey test table for effect of pressure on the MS resistance of PME in orange juice
(Dependent variables: Log D_{MS} values)

Pressure	N	Subset		
	1	2	3	1
200	3	7,6867		
100	3		25,2900	
0	3			35,2567
Sig.		1,000	1,000	1,000

Table C9. ANOVA table for effect of temperature on the TT resistance of PPO in apple juice
(Dependent variables: Log D_{TT} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1438003,462(a)	3	479334,487	76,718	,000
Intercept	639443,882	1	639443,882	102,344	,000
Temperature	1438003,462	3	479334,487	76,718	,000
Error	49983,761	8	6247,970		
Total	2127431,105	12			
Corrected Total	1487987,222	11			

a R Squared = ,966 (Adjusted R Squared = ,954)

Table C10. Tukey test table for effect of temperature on the TT resistance of PPO in apple juice
(Dependent variables: Log D_{TT} values)

temperature	N	Subset	
	1	2	1
60	3	5,4690	
50	3	19,8467	
40	3	69,0100	
25	3		829,0333
Sig.		,762	1,000

Table C11. ANOVA table for effect of temperature on the TT resistance of PME in orange juice
(Dependent variables: Log D_{TT} values)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1537197,785(a)	4	384299,446	451,246	,000
Intercept	811518,140	1	811518,140	952,888	,000
Temperature	1537197,785	4	384299,446	451,246	,000
Error	8516,408	10	851,641		
Total	2357232,333	15			
Corrected Total	1545714,193	14			

a R Squared = ,994 (Adjusted R Squared = ,992)

Table C12. Tukey test table for effect of temperature on the TT resistance of PME in orange juice
(Dependent variables: Log D_{TT} values)

temperature	N	Subset		
	1	2	3	1
70	3	23,3267		
60	3	36,8300		
50	3	48,9600		
40	3		192,8000	
25	3			861,0667
Sig.		,815	1,000	1,000

Table C13. ANOVA table for effect of temperature on MTS inactivation of PPO in apple juice at 200 kPa constant pressure and 110 μm constant amplitude.
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	12,608(a)	5	2,522	191,907	,000
Intercept	54,548	1	54,548	4151,359	,000
Pressure	3,491	1	3,491	265,707	,000
Amplitude	,000	0	.	.	.
Temperature	8,341	2	4,170	317,384	,000
Pressure * amplitude	,000	0	.	.	.
Pressure * temperature	,776	2	,388	29,530	,000
amplitude * temperature	,000	0	.	.	.
Pressure * amplitude * temperature	,000	0	.	.	.
Error	,158	12	,013		
Total	67,313	18			
Corrected Total	12,766	17			

a R Squared = ,988 (Adjusted R Squared = ,983)

Table C14. Tukey test table for effect of temperature on MTS inactivation of PPO in apple juice at 200 kPa constant pressure and 110 μm constant amplitude.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60	6	,9071		
55	6		1,7408	
50	6			2,5745
Sig.		1,000	1,000	1,000

Table C15. ANOVA table for effect of temperature on MTS inactivation of PPO in apple juice at 200 kPa constant pressure and 110 μm constant amplitude.
(Dependent Variable: D_{MTS} values).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	138,717(a)	2	69,359	26,600	,001
Intercept	713,727	1	713,727	273,724	,000
Temperature	138,717	2	69,359	26,600	,001
Error	15,645	6	2,607		
Total	868,089	9			
Corrected Total	154,362	8			

a R Squared = ,899 (Adjusted R Squared = ,865)

Table C16. Tukey test table for effect of temperature on MTS inactivation of PME in orange juice at 200 kPa constant pressure and 110 μm constant amplitude.
(Dependent Variable: D_{MTS} values).

temperature	N	Subset		
	1	2	3	1
60	3	4,0673		
55	3		8,9650	
50	3			13,6833
Sig.		1,000	1,000	1,000

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Güzel, Burçin Hülya
Nationality: Turkish(TC)
Date and Place of Birth: 24 May 1981, Istanbul
e-mail: burcingzl@gmail.com

Education

- **Doctor of Philosophy (Integrated Ph. D. Program,** applying with a Bachelor's degree), Food Engineering Department, Middle East Technical University (METU), Ankara, 2006-2013.
- Bachelor of Science, Food Engineering Department, Ankara University, Ankara, 1998-2002

Research Experience

- Research Assistant, Food Engineering Department, Middle East Technical University (METU), Ankara, 2004-2013.
- Research Scholar, Tecnología de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain, June 2010-February 2011 (Supervised by Prof. Dr.Santiago Uson Condón).

Project : M.E.T.U BAP-08-11-DPT.2002K120510, Researcher, 2006-2013.

Publications

Guzel, B H, Arroyo, C, Condón, S, Pagán, R, Bayındırlı, A & Alpas, H. (2013). Inactivation of *Listeria monocytogenes* and *Escherichia coli* by Ultrasonic Waves Under Pressure at Nonlethal (Manosonication) and Lethal Temperatures (Manothermosonication) in Acidic Fruit Juices. Food and Bioprocess Technology: An International Journal.

Guzel, B H, Arroyo, C, Condón, S, Pagán, R, Bayındırlı, A & Alpas, H. (2013). Inactivation of Polyphenoloxidase and Pectinmethlyesterase by Ultrasonic Waves Under Pressure at Nonlethal (Manosonication) and Lethal Temperatures (Manothermosonication) in Acidic Fruit Juices (submitted)