Unconventional technologies to steer food quality and functionality

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Preface

Nowadays, food processing is aimed to develop foods that are not only safe and nutritious, but also characterized by specific technological properties (structural, stabilizing, sensory) and health promoting functions. These important food functions, however, are strongly dependent on the way the foods are processed.

Unconventional technologies, such as ultrasounds, high pressure, pulsed electric fields, high pressure carbon dioxide and UV light, have been investigated in order to satisfy the demand for foods able to accomplish desired technological and functional properties. However, in this context, the understanding of the mechanism inherent to the process and the selection of process parameters are crucial.

This Ph.D. thesis aimed to investigate the effects of unconventional technologies on food quality and functionality of fruit and vegetable derivatives. To this purpose, selected unconventional technologies such as ultrasounds, high pressure homogenization and pulsed electric fields were applied and their effects on physical and chemical properties of fruit and vegetable derivatives and on *in vitro* functionality of bioactive compounds were investigated.

Chapter 1

Food quality and functionality of fruit and vegetable derivatives

Food quality is a multidimensional concept which includes extrinsic and intrinsic factors (Trijp, & Steenkamp, 1998). The extrinsic factors are inherent to the way food is produced such as the use of pesticides, the absence of child labour, animal-friendliness or use of genetically modified organisms. The intrinsic factors are associated to food safety, physical (texture, flavour, taste) and health related properties (Linnemann et al., 2006). During the last decades, health related properties have become more and more important due to lifestyle change, increasing cost of healthcare and life expectancy as well as the desire for improved quality of life (Kotilainen et al., 2006). For these reasons, innovation in the food sector is now directing towards strategies for ensuring high nutritional and functional properties of foods.
Among the food products, fruit and vegetable derivatives are recommended by dietary guidelines due to their high content of nutrients such as vitamins, minerals, fibres, carotenoids and polyphenols. These compounds prevent many chronic disease associated with cancer, inflammation, atherosclerosis and aging caused by free radicals (Liu, 2003; Liu, 2004). However, most of the fruit and vegetable derivatives have to be processed before the consumption in order to ensure food safety, quality and worldwide supply independently from their seasonal availability.

1.1 Conventional food processing

Food processing can be defined as a set of unit operations by which raw foodstuffs are made suitable for consumption, cooking or storage. The main purpose is to assure safe food and maintain as long as possible desired quality and functional properties. Food processing encompasses a large variety of processing such as fermentation, heating, drying, smoking, mechanical processes, cooling and freezing. Among them, heating is extensively used in food technology. Since prehistoric time, our ancestors learned how to master the fire through a trial and error approach and it is widely accepted that cooking had an evolutionary effect because it increased the food nutritional value and allowed to human ancestors to spend less time foraging, chewing and digesting. As a consequence, our ancestors developed a smaller and more efficient digestive apparatus which freed up energy to enable larger brain growth (Wrangham, 2009). Moreover, the fire discovery and the continuous development of thermal treatment had a very high impact on phenotypical, intellectual, societal and economic development of human being (Laden et al., 2006). During the centuries, the use of heat changed from domestic use to more industrialized process with the first sterilization plant. Modern thermal processing was developed in the nineteenth and twentieth centuries to serve military needs. In particular, in 1809 Nicolas Appert invented a vacuum bottling techniques to supply French troops. Later on, in 1862, Louis Pasteur discovered the lethal effect of heat on microorganism with the pasteurization treatment (Floros et al., 2010; Norton, Fryer, & Moore, 2006).

It is well known that the foremost beneficial effects of thermal treatment are microbial decontamination, enzyme and toxins inactivation (van Boekel et al., 2010). Next to these effects, thermal treatments cause denaturation and gelatinization of protein and starch, respectively, leading to an increase of their digestibility (Linnemann et al., 2006). Moreover, heating causes the disruption of cell membrane and β-eliminative pectin degradation in plant cells walls (Greve et al., 1994). Thermal treatments lead to the formation of desired compounds, such as flavour, colouring and health promoting compounds. However, some detrimental effects can occur such as losses of important food nutrients (i.e. vitamin C, polyphenols) (Nayak, Liu, & Tang, 2015).

1.2 Unconventional food processing

Since the beginning of the last century, unconventional technologies have gained more and more interest in the attempt to reduce, at least partially, the negative effects of thermal treatment and, at the same time, ensure safe food products (Raso & Barbosa-Cánovas, 2003).

Unconventional technologies are a group of technologies based on driven forces different from heating. For instance, mechanical, electromagnetical, chemical stresses occur during the application of ultrasounds, high pressure, pulsed electric fields, UV light, high pressure carbon dioxide. The main purpose of unconventional technologies is to obtain safe foods, however, any process used to kill microorganisms might affect the physical and chemical properties of the food products. For this reason, nowadays, the challenge for food scientists is to understand the impact of unconventional technologies in order to steer food products with high quality and functionality.

Among the unconventional technologies, the most promising ones appear to be ultrasound (US), high pressure homogenization (HPH) and pulsed electric fields (PEF). Their effects on microbial decontamination and enzyme inactivation have been extensively investigated in model and food systems, while data available on their influence on the functional properties of fruit and vegetable derivatives are still limited.

Hereafter, an overview of the effects of US, HPH and PEF treatments on the main quality and functional properties is presented.

1.2.1. Effect of US treatments on quality and functional properties of fruit and vegetable derivatives

US are acoustic waves at frequency higher than 18 kHz. The main phenomenon occurring during US treatment is cavitation, which consists in the formation and violent collapse of small bubbles, generating shock waves associated to high local temperatures (1000-5000 K) and pressures (100-50000 bar) inside the collapsing bubbles (Leighton, 1994). These extreme conditions lead to the occurrence of physical (microject, turbulence, shear forces) and chemical phenomena (formation of free radicals) (Gogate, Wilhelm, & Pandit, 2003).

US treatments are inefficient in achieving 5 log reduction as required by the Food and Drug Administration (USFDA, 2000). In fact, US treatments allow to reduce by 2 log microbial population in fruit and vegetable juices (Elizaquível et al., 2012; Gómez-

López et al., 2014; Gómez-López et al., 2015). Similarly, US treatments are inefficient to inactivate enzymes within a reasonable treatment time for industrial application (Terefe, Buckow, & Versteeg, 2015b). Therefore, current research is focusing on the combination of US with conventional or other unconventional technologies to increase its effectiveness. For instance, US treatments applied in combination with heating (up to 90 °C) and/or pressure (up to 600 MPa), chlorine or a commercial mixture of organic acids and phenolic compounds resulted more effective in killing microorganisms and inactivating enzymes (Gómez-López et al., 2014; Gómez-López et al., 2015; Lee et al., 2013; Madge & Jensen, 2002; Mason et al., 2003; Terefe et al., 2015b; Ugarte-Romero et al., 2006).

In addition to microbial and enzyme inactivation, US treatments have been applied for the development of food products able to accomplish desired technological and nutritional functions. For instance, low frequency (<100 kHz) ultrasound processing may induce changes in the viscoelastic properties of fruit and vegetable derivatives due to the disruption of plant cell membranes and release of intracellular compounds (Anese et al., 2013; Rojas et al., 2016; Wu et al., 2008). Such structural modifications have been suggested to affect the bioaccessibility of carotenoids in tomato. In particular, Anese et al. (2013) found that ultrasounds, while causing a great increase in viscosity, induced a marked decrease in *in vitro* lycopene bioaccessibility. On the other hand, high frequency US (>100 kHz) treatments may modify the antioxidant properties of bioactive molecules due to the production of hydroxyl radicals (Ashokkumar et al., 2008).

1.2.2. Effect of HPH treatments on quality and functional properties of fruit and vegetables derivatives

During HPH process, a fluid, which is pumped through a narrow gap valve by pressure intensifier, undergoes intense mechanical forces and elongational stresses at the valve entrance and in the valve gap, while turbulence, cavitation and impacts with the solid surface occur at the gap outlet (Floury et al., 2004a; Floury et al., 2004b). Under these conditions, the fluid is also accompanied by short-life heating phenomena ranging between 0.5-0.2 °C/MPa (Datta et al., 2005).

HPH processing has been extensively studied as a tool for microbial inactivation in fruit and vegetable juices as well as model systems (Maresca, Donsi, & Ferrari, 2011; Patrigani & Lanciotti, 2016). Generally, the process efficiency depends on pressure level, number of passes through the homogenization valve as well as characteristics of microorganisms and medium (Donsì et al., 2009). By applying increasing pressure (up to 150 MPa) and number of passes, no significant reduction in microbial population has been found in model systems as well as fruit and vegetable juices. To achieve 5 log reductions of microbial population, pressure values higher that 200 MPa have to be applied (Patrigani & Lanciotti, 2016).

The effect of HPH treatments on enzyme activity has been evaluated by several authors (Lacroix, Fliss & Makhlouf, 2005; Tribst, & Cristianini, 2012; Welti-Chanes, Ochoa-Velasco & Guerrero-Béltran, 2009). At pressures lower that 200 MPa, slight or no changes in enzymatic activity were observed (Tribst & Cristianini, 2012). Pressures values above 200 MPa induced the inactivation of the main enzymes responsible for

quality loss in fruit and vegetable derivatives (Suarez et al., 2010; Welti-Chanes et al., 2009).
In addition to microbial and enzyme inactivation, HPH processing is also widely used to produce fine and stable nanoemulsions in the pressure range between 50 and 150 MPa (Dumay et al., 2013). Moreover, the effect of HPH processing has been also evaluated to modify the physical properties of particles suspended in a fluid. In particular, HPH can affect the rheological properties of fruit and vegetable juices due to cells disruption with leakage of plant constituents, including biopolymers, in the serum (Kubo et al., 2013; Colle et al., 2010b). It has been reported that HPH processing can modify physical properties of fruit and vegetable juices, such as tomato, pineapple, banana, mango, pineapple, peach, tomato, broccoli, carrot juices (Bengtsson & Tornberg, 2011; Lopez-Sanchez et al., 2011a; Lopez-Sanchez et al., 2011b; Silva et al., 2010). Moreover, it has been suggested that modification in physical properties may affect the release of micronutrients from the food matrix and thus limit their bioaccessibility. For instance, carotenoids bioaccessibility in tomato juice decreased upon HPH treatments due to the formation of a network that limits the carotenoids release from the matrix (Colle et al., 2010b; Panozzo et al, 2013).

1.2.3. Effect of PEF treatments on functional properties of fruit and vegetable derivatives

PEF is a widely explored technology for inducing permeabilization of cell membranes. The exposure of a cell to an electric field for short voltage pulses, typically in the range of μs, induces the formation of pores on the membrane (electroporation phenomena). More specifically, when the cells are exposed to an external electric field, the accumulation of oppositely charged ions on both sides of membrane causes membrane thickness reduction. Further increases of the electric field up to critical values cause pore formation and loss of semi-permeability of the cell membranes. However, depending on electric field strength and treatment intensity, electroporation may be either reversible or irreversible (Zimmermann, 1986).
PEF treatments at electric field intensity higher than 10 kV/cm for short pulses are effective to inactivate spoilage as well as pathogenic microorganisms that may represent a public health risk (*L. monicytogenes, S. aureus, E. coli* and *S. Typhimurium*) (Bermudez-Aguirre, Dunne, & Barbosa-Cánovas, 2012; Saldaňa et al., 2014). On the contrary, any lethal effect has been observed in spores due to the spores'envelopes that prevent the permeabilization of cytoplasmatic membrane (Grahl & Markl, 1996; Pagan et al., 1998; van Heesch et al., 2000; Katsuki et al., 2000). Moreover, the effect of PEF on enzyme inactivation is often limited under sufficient condition for microbial inactivation (Terefe, Buckow, & Versteeg, 2015a). Therefore, in order to increase the efficiency of PEF towards microbial decontamination and enzyme inactivation various strategies have been suggested. For instance, the increase of inlet temperature, electric field strength, treatment time or reduction of pH resulted in an improved inactivation level (Barbosa-Cánovas et al., 1999; Wouters & Smelt, 1997; Wouters et al., 1997). Furthermore, PEF treatments in combination with high hydrostatic pressure, high pressure carbon dioxide, ultrasound treatments and ultraviolet radiation have been

proven to be effective in increasing microbial decontamination (Martin-Belloso & Sobrino-Lopez, 2011). Under these process conditions, PEF treatments allowed to maintain the nutritional quality and the antioxidant content of fruit and vegetable juices (Soliva-Fortuny et al., 2009).

PEF treatments at electric field strength lower than 5 kV/cm are applied to induce cell permeabilization of plant tissue and thus enhance the mass transfer of inner liquid and cell components from the plant cells (Donsì, Ferrari, & Pataro, 2010). In particular, several studies showed that PEF treatment can be used as a pre-treatment to facilitate drying and osmotic dehydration (Angersbach, Heinz, & Knorr, 2000). In addition, PEF treatments are applied individually or in combination with heating in order to improve the extraction yield of intracellular compounds present in fruit and vegetable tissue (Donsì et al., 2010). Several studies found that PEF treatments increased the extraction of hydrophilic compounds, such as sugar from sugar beet, betaine from red beet and anthocyanins from grapes, red cabbage or purple fleshed potatoes (Eshtiaghi & Knorr, 2002; Fincan, DeVito, & Dejmek, 2004; Gachovska et al., 2010; López et al., 2009; Puértolas et al., 2013). By contrast, only a few studies investigated the effect of PEF treatments on the extraction of lipophilic compounds, such as carotenoids (Luengo, Álvarez, & Raso, 2014; Wiktor et al., 2015).

1.3 Research needs

Despite the huge number of studies available in the literature on the effect of unconventional technologies, there is conflicting information regarding the effects of unconventional technologies on quality and functional properties of fruit and vegetable derivatives. This can be mainly attributed to poor descriptions of the operating protocol and lack of standardization of process conditions. For instance, frequency, amplitude, nominal power, energy input and temperature evolution during the process, need to be specified to fully describe the US process. In particular, temperature represents a very important process parameter during US treatment because the acoustic energy is ultimately converted to heat. Therefore, next to the mechanical effect, the temperature increase has to be considered (Kentish & Ashokkumar, 2011). During HPH treatment, valve geometry, flow conditions and final temperature are the main process parameters. Similarly to US treatment, an important temperature rise, that may affect the sensory and nutritional properties of processed food, can be observed in the fluid downstream of the valve (Floury et al., 2000). During PEF treatments, peak voltage, pulse shape, number of pulses, width, chamber characteristics, temperature and physical-chemical characteristics of the medium need to be reported in order to describe the process.

All these information are also essential for the reproducibility of the experiment in view of definition of process conditions in commercial size equipment. In fact, it should be underlined that most of the studies are based on laboratory scale-machine and it is well known that the effectiveness of the process is heavily scale dependent and that the results cannot be readily transferred to industrial level.

Moreover, unconventional technologies have been also proposed as technologies able to reduce energy and water consumption; therefore they can play a role toward economic and environmental sustainability of food processing.

Based on these evidences, further research on the effect of unconventional technologies could include: (a) identification and harmonization of the key information that should be reported in order to avoid ambiguity among the results and favour the understanding of the mechanisms inherent to the process; (b) identification of a reference parameter that takes into account different process parameters; (c) scaling-up of the process in view of the industrial application; (d) estimation of the energy efficiency and environmental impact.

Finally, to meet industries' and consumers' expectations for foods with specific health benefits, the nutritional functions have to be tested. Several studies investigated the effect of HPH on functional properties of bioactive compounds naturally present in tomato juice (Colle et al., 2010b; Panozzo et al., 2013; Palmero et al., 2014). On the other hand, only few data are available on the effect of US and PEF treatments on functional properties of fruit and vegetables derivatives (Anese et al., 2013; Jayathunge et al., 2017). Moreover, most of the fruit and vegetable derivatives are used as ingredients in formulated products or stored. However, available data on the effect of unconventional technologies on functionality of fruit and vegetable derivatives added or not with other ingredients (i.e. oil) or during storage are lacking. In this context, further research is needed to investigate the effect of unconventional technologies on functional properties of fruit and vegetables derivatives.

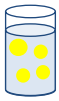
1.4. Aim and outline of this Ph.D. thesis

This Ph.D. thesis aimed to investigate the potentials of unconventional technologies to steer food quality and functionality in fruit and vegetable derivatives. To this aim, US, HPH and PEF were selected as unconventional technologies.
In particular, the research was divided into two parts (Table 1.1).

In the first part, the effect of HPH, US and their combinations on food quality was investigated. In Chapter 2 the influence of HPH, low frequency US and their combinations on polyphenoloxidase activity in apple juice was studied. Chapter 3 aimed to investigate the effect of HPH and low frequency US treatments on some physical properties (viscoelastic properties, Bostwick consistency, precipitate weight ratio, pectin esterification degree and microstructure) of tomato juices with different soluble solids contents (5.0, 7.5, 10.0 °Brix). Finally, in order to ensure high microbiological quality of wastewater deriving from fresh-cut vegetables washing and optimize wastewater management, Chapter 4 was addressed to investigate the effect of low frequency US on microbial decontamination of wastewater.

In the second part, the effect of HPH, US and PEF on functional properties of fruit and vegetable derivatives was studied. In Chapter 5, HPH treatment was applied to produce an oil-in-water nanoemulsion enriched with silymarin, a mixture of bioactive compounds (i.e. silybin, silydianin and silychristin) extracted from *Silybum marianum*. Physical stability of nanoemulsions, chemical stability and silybin *in vitro* bioaccessibility were investigated. Chapter 6 aimed to study the effect of high frequency US on functional properties of tomato juice. Furthermore, in Chapter 7, the effect of low frequency US treatment on tomato juice with or without a lipid phase, on lycopene concentration and *in vitro* bioaccessibility at time zero and during storage was investigated. In Chapter 8, the effect of PEF treatment on carotenoids (β-carotene and lycopene) bioccessibility in tomato fractions with different structural barriers was studied.

Table 1.1 Overall research of this Ph.D. thesis.

	Technology	Matrix	Effect
Food quality	HPH and/or US	 Apple juice	✓ Polyphenoloxidase activity
	US or HPH	 Tomato juice	✓ Physical properties
	US	 Wastewater from lettuce washing	✓ Microbial decontamination
Food functionality	HPH	 Nanoemulsion enriched with silymarin	✓ Physical and chemical stability ✓ Silybin bioaccessibility
	US	 Tomato juice	✓ Carotenoids and polyphenols concentration ✓ α-glucosidase inhibitory activity
	US	 Tomato juice	✓ Carotenoids stability ✓ Carotenoids bioaccessibility
	PEF	 Tomato fractions	✓ Carotenoids bioaccessibility

Part I

Effect of unconventional technologies on quality of fruit and vegetable derivatives

Chapter 2

Effect of HPH, low frequency US treatments and their combinations on polyphenoloxidase activity in apple juice

Despite a number of studies have been reported on the effects of HPH and low frequency US treatments on food quality-related enzymes (Liu at al., 2009a; Liu et al., 2009b; Suarez-Jacobo et al., 2012), the results are often contradictory. In fact, both activation and inactivation effects on polyphenoloxidase (PPO) in fruit juices and model systems subjected to HPH or US are described, due to differences in equipment, process conditions, enzyme source, etc. (Costa et al., 2013; Liu et al., 2009a; Liu et al., 2009b; Silva et al., 2015; Suarez-Jacobo et al., 2012 Yu, Zeng, & Lu, 2013). Moreover, PPO inactivation can be obtained by applying intense HPH and US processes conditions (i.e. high pressures/number of passes and long ultrasonication time) (Suarez-Jacobo et al., 2012; Abid et al., 2014). It is noteworthy that these process conditions might not fit the industrial needs as they can contribute to increasing the total cost of ownership.

2.1 Aim of the study

The aim of this study was to investigate the influence of individual and combined HPH and low frequency US treatments on PPO activity in apple juice. To this purpose, apple juice was subjected to individual and combined HPH and US with or without temperature control and the residual PPO activity was evaluated. The final aim was to find the potentiality of combined HPH and US treatments with or without temperature control in the attempt to reduce energy density.

2.2. Materials and methods

2.2.1. Preparation of apple juice

A 20 kg batch of fresh apples (*Malus domestica* Borkh., cv. Golden Delicious) was purchased at the local market and maintained at 7 °C until use. Apple juice was prepared fresh for every trial from the same batch of fruits to minimize sample variability. The apples were peeled and the juice was extracted using a household table top juice extractor (Ariston Hotpoint Slow Juicer, Fabriano, Italy). The extract was filtered through a filter cloth to remove impurities and coarse particles, centrifuged at 4000 g for 5 min at 4 °C (Beckman Avanti tm J-25, Beckman Instruments Inc., Palo Alto, CA, USA) and filtered again by using a filter cloth. The resulting clear apple juice having a soluble solid content of 5 ± 1 °Brix, a pH of 3.6 ± 0.2 was immediately subjected to HPH and/or US with or without temperature control.

2.2.2. Polyphenoloxidase model solution

Mushroom (*Agaricus bisporus)* tyrosinase (polyphenoloxidase, PPO) (T3824 50 KU, Sigma-Aldrich, St Louis, MO, USA) was used. PPO model solution was prepared by diluting the enzyme in 0.1 M potassium phosphate buffer at pH 7.0 (Carlo Erba, Milano,

Italy). PPO initial activity in 1.5 10^{-3} M 2,4 dihydroxy-L-phenyl-alanine (L-DOPA, Sigma-Aldrich, St Louis, MO, USA) was 0.005 Abs/min, and comparable to that of the PPO in apple juice. The model was subjected to similar processes as the apple juice was.

2.2.3. Treatments

2.2.3.1. HPH

A continuous lab-scale high-pressure homogenizer (Panda Plus 2000, GEA Niro Soavi S.p.a., Parma, Italy) supplied with two Re+ type tungsten carbide homogenization valves, with a flow rate of 2.5 cm^3/s, was used. In particular, the first valve was the actual homogenization stage and was set at increasing pressures from 20 to 150 MPa. The second valve was set at the constant value of 5 MPa. Additional samples were prepared by subjecting the apple juice to HPH for up to 10 successive passes at 150 MPa. At the exit of the homogenizer, the sample was forced into a heat exchanger (GEA Niro Soavi, S.p.a., Parma, Italy) and cooled to 20 ± 2 °C. Then, after the final pass in the homogenizator, it was cooled in a water-ice bath.

2.2.3.2. Low frequency ultrasound treatment with (US) and without (US-TT) temperature control

An ultrasonic processor (Hieschler Ultrasonics GmbH, mod. UP400S, Teltow, Germany) with a titanium horn tip diameter of 22 mm was used. The instrument operated at constant ultrasound amplitude and frequency of 100 µm and 24 kHz, respectively. During the ultrasonication experiment, the temperature was either controlled using a cryostatic bath, to dissipate the heat generated during treatment, or uncontrolled, leaving the temperature to rise due to heat dissipation. Aliquots of 150 mL of sample were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass vessel. The tip of the sonicator horn was placed in the centre of the fluid, with an immersion depth of 25 mm. The ultrasound treatments were performed for increasing time periods up to 45 and 7 min for the controlled (US) and uncontrolled (US-TT) temperature regimes, respectively. Following the treatments, the sample was refrigerated in a water-ice bath.

2.2.3.3. Combination of high pressure homogenization and ultrasonication with (HPH-US) and without (HPH-US-TT) temperature control

The apple juice was subjected to HPH at 150 MPa followed by US treatment with (HPH-US) or without (HPH-US-TT) temperature control for increasing time periods. Samples were cooled in a water-ice bath at the end of the second treatment.

2.2.3.4. Thermal treatment

The total temperature-time combination received by the sample during ultrasonication was applied to the sample in the absence of the ultrasound treatment. To this purpose, aliquots of 150 mL of apple juice or PPO solution were introduced into 250 mL capacity glass vessel and heated in a thermostatic water bath (Ika Werke, MST BC, Staufen, Germany) under continuous stirring, by mimicking the same temperature profile

produced during US treatment with (TT_c) and without (TT) temperature control. Following the treatments, the sample was refrigerated in a water-ice bath.

2.2.4. Temperature measurement

The sample temperature was measured just before and immediately after (i.e. before the cooling step) each treatment by a copper-constantan thermocouple probe (Ellab, Hillerød, Denmark) immersed in the fluid, connected to a portable data logger (mod. 502A1, Tersid, Milan, Italy). In addition, during US and thermal treatments, the temperature was recorded as a function of time, by immersing the thermocouple tip in the fluid, half way between the solution centre and the inside wall of the vessel.

2.2.5. Energy density computation

The energy density (E_v, MJ/m^3) transferred from the homogenization valve to the sample was determined as described by Stang, Schschmann and Schubert (2001), according to eq. 2.1:

$$E_v = \Delta P \qquad \text{(eq. 2.1)}$$

where ΔP is the pressure difference operating at the nozzles.

The power density (P_v, W/ m^3) transferred from the ultrasound probe to the sample was determined calorimetrically by recording the temperature (T, K) increase during the treatment, following eq. 2.2 (Raso et al., 1999).

$$P_v\,(T) = mc_p(\partial T/\partial t)/V \qquad \text{(eq. 2.2)}$$

where m is the sample mass (kg), c_p is the water heat capacity (4.18 kJ/kg K), V is the sample volume (m^3), and t (s) is the time frame of treatment considered. In order to achieve at least an estimation of the energy density over the whole treatment while including the effect of temperature, the power density was measured as a function of temperature for a separate test with thermal insulation and without temperature control. Later on, the energy density (MJ/m^3) was estimated by integration of the power density as:

$$E_v = \int P_v\,(T)\,dt = \sum(P_v(T)\Delta t) \qquad \text{(eq. 2.3)}$$

on the whole treatment time.

The energy density of multiple passes HPH and combined treatments was calculated as the sum of the energy density values of the corresponding single pass HPH, or US with or without temperature control plus HPH treatments (Calligaris et al., 2016).

2.2.6. Apple juice soluble solids content and pH

Soluble solid content was measured using a table refractometer (Unirefrax, Bertuzzi, Milan, Italy) calibrated with distilled water. The pH was measured at 25 °C using a pHmeter (Crison, model 2002, Alella, Spain) equipped with a combination of glass electrodes and a temperature probe.

2.2.7. Determination of PPO activity

The PPO activity was determined spectrophotometrically (Shimadzu UV-2501PC, UV-Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 25 °C (Kahn, 1985). Aliquots of 0.5 mL of apple juice were added to 2.5 mL of 1.5 10^{-3} M L-DOPA. Additional trials were performed by adding 0.4 mL of PPO solution to 2 mL of 1.5 10^{-3} M L-DOPA. The absorbance at 420 nm was monitored each minute for 10 min. The changes in absorbance per min were calculated by linear regression, applying the pseudo zero order kinetic model. When present, the final stationary phase was excluded from regression data. One arbitrary unit of PPO was defined as the amount of enzyme that produced an increase in absorbance at 420 nm of 0.001 Abs/min under the testing conditions. PPO activity (%) was calculated as the percentage ratio between the rate constants (Abs/min) of the enzymatic activity of the treated and untreated samples.

2.2.8. SDS-polyacrilamide gel electrophoresis

Sodium dodecyl sulphate polyacrilammide gel electroforesis (SDS-PAGE) was performed according to Laemmli (1970). Aliquots of PPO solution in 0.1 M potassium phosphate buffer pH 7.0 were introduced into centrifugal filters (Vivaspin 500, 5KMWCO, Sartorius Stedim Biotech GmbH, Goettingen, Germany) and ultracentrifuged 4 times at 13000 rpm (MiniSpin plus, Eppendorf, Milan, Italy) for 10 min. Aliquots of concentrated sample were added to sample buffer containing 0.01% bromophenol blue, 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) β-mercaptoethanol. The sample was loaded on a 13% (w/v) polyacrylamide separating gel containing 0.4% (v/v) bis-acrylamide cross linker, mounted on a Mini 2-D (Bio-Rad, Richmond, California, USA) electrophoresis cell. The electrophoresis separation was obtained by running the sample at 15 mA and 150 V with standard proteins of known molecular weight (Precision Plus Protein™ Dual Color Standards, Bio-Rad, Segrate, Italy). The gels were subsequently stained with NOVEX colloidal Blu Staining Kit (Invitrogen, Carlsbad, California, USA) for one night and destained in bi-distilled water. Gels were scanned and analysed with the ImageQuant TL Image Analysis Software (Amersham Bioscience INc., Piscataway, New Jersey, USA). Estimation of the molecular weight of each band was performed by interpolating its migration distance with those of protein standards.

2.2.9. Data analysis

The results are the average of at least three measurements carried out on two replicated experiments ($n \geq 6$). Data are reported as mean value ± standard error. Statistical analysis was performed using R v.2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA was carried out

and Tukey test was used to determine statistically significant differences among means (p<0.05). Linear regression analysis of PPO activity (%) of apple juice subjected to treatments *vs* energy density (MJ/m^3) was performed by using Microsoft Excel 2013. Rate constants (k) were computed from the slopes of the linear regression. The goodness of fitting was evaluated based on visual inspection of residual plots and by calculation of R^2 and p. The acoustic contribution to PPO inactivation was computed as the percentage ratio of the rate constants of US with and without temperature control ($k_{US}/k_{US\text{-}TT} \cdot 100$); the thermal contribution was calculated as the percentage ratio of the rate constants of thermal and US processes with or without temperature control ($k_{TTc}/k_{US} \cdot 100$ and $k_{TT}/k_{US\text{-}TT} \cdot 100$, respectively).

2.3. Results and discussion

The effects of HPH, US, and combinations of HPH and US (HPH-US) on PPO activity of freshly prepared apple juice were evaluated. The effect of *in situ* generated heat during US without temperature control (US-TT) and combinations of HPH and US (HPH-US-TT) on enzyme activity was also considered. In order to compare the effect of these technologies, the energy density was taken as a reference parameter. This parameter is an indicator of the treatment intensity because it incorporates the transferred power, the duration of the treatment and the treated sample volume (Hulsmans et al., 2010; Stang, Schschmann, & Schubert, 2001).

Table 2.1 shows the maximum temperature and the energy density of the samples subjected to the treatments. During HPH, temperature increased linearly with the increasing of pressure ($R^2 > 0.99$, $p < 0.05$) or number of passes ($R^2 > 0.89$, $p < 0.05$) up to 56 °C, and the energy density ranged between 50 and 1500 MJ/m^3. Comparable energy densities were obtained for US treatments performed under controlled temperature regime, during which temperature never exceeded 42 °C. When performed without temperature control, US-TT treatment was responsible for a linear ($R^2 > 0.93$, $p < 0.05$) temperature increase up to 78 °C and the energy densities transferred from the probe into the fluid ranged between 49 MJ/m^3 and 346 MJ/m^3. Combinations of HPH (150 MPa, single pass) and US (up to 15 min), with (HPH-US) or without (HPH-US-TT) temperature control, for short lengths of sonication time gave energy densities comparable to those generated by the application of intense individual HPH and US treatments. For instance, an energy density of approximately 350 MJ/m^3 was obtained by applying 10 min US, 7 min US-TT, or 4 min HPH-US-TT.

Table 2.1. Temperature and energy densities of apple juice subjected to HPH and/or US treatments. Indication of US process performed with or without temperature control is also given. Starting temperature 8.0 ± 1.0 °C.

Treatment	Temperature control	Pressure (MPa)	Number of passes	Time (min)	Temperature (°C)	Energy density (MJ/m^3)
HPH	no	50	1		27.5±2.3	50
		100	1		35.6±1.7	100
		150	1		42.6±1.2	150
		150	3		44.7±1.2	450
		150	5		51.6±3.0	750
		150	8		52.4±0.9	1200
		150	10		56.4±0.6	1500
US	yes			5	38.4±1.6	164
				10	41.6±2.3	328
				15	40.8±1.6	492
				30	40.4±0.4	984
				45	41.9±2.7	1476
HPH-US	yes	150	1	2	41.3±1.1	150
		150	1	3	41.3±3.3	216
		150	1	4	44.9±1.4	281
		150	1	5	46.2±1.3	314
		150	1	10	47.4±1.8	478
		150	1	15	46.7±1.2	642
US-TT	no			1	28.4±3.3	49
				2	42.2±4.0	99
				3	55.6±3.7	148
				4	64.6±4.8	198
				5	70.3±2.9	247
				6	73.9±2.6	297
				7	77.9±1.2	346
HPH-US-TT	no	150	1	2	58.4±2.3	256
		150	1	3	67.0±4.8	309
		150	1	4	73.9±5.8	362

Figure 2.1 shows the effect of HPH and US treatments on PPO activity of apple juice as a function of energy density. No significant reduction of PPO activity was achieved by applying HPH in the energy density range between 50 MJ/m^3 and 150 MJ/m^3, corresponding to a single pass at pressures increasing from 50 to 150 MPa. Only an increase in energy density from 150 to 1500 MJ/m^3, obtained by submitting the apple juice to multiple passes through the HPH valve, allowed PPO activity to be decreased, reaching a residual activity of 50%. Either activation or inactivation effects have been reported in the literature for HPH pressures ranging from 80 to 300 MPa (Liu et al., 2009a; Liu et al., 2009b; Suarez-Jacob et al., 2012). In particular, the PPO inactivation has been attributed to loss of the native structure due to temperature increase and mechanical forces generated by the passage of the fluid through the homogenization valve. In our experimental conditions, the modest temperature increase (up to 40 °C) together with the short residence time (approximately 10^{-4} s) in the homogenization

valve (Jafari, He, & Bhandari, 2007) may be responsible for the inefficacy of single-pass HPH treatments in inactivating PPO. On the contrary, the efficacy of multiple HPH passes in reducing PPO activity can be attributed to the increase in shear stresses, cavitation and turbulence, as well as to the multiplication of treatment time by the number of passes and to the higher temperature reached (up to 57 °C after 10 passes at 150 MPa; Table 2.1). To this regard, it has been reported that mushroom PPO remained fully active up to 40 °C, whereas inactivation occurred at temperatures between 50 °C and 70 °C (Baltacioğlu et al., 2015).

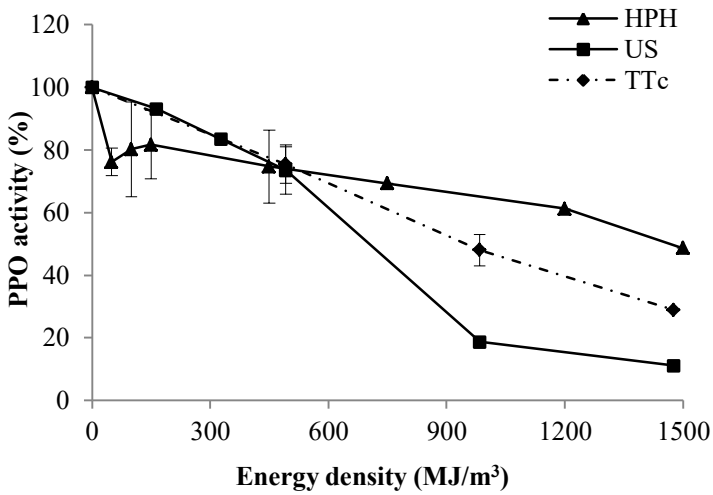


Figure 2.1. PPO activity of apple juice subjected to individual HPH and US treatments as function of energy density. TT_c: heat treatment obtained by providing the sample the same time-temperature combinations received during US with temperature control.

The application of US at moderate energy density values (up to 492 MJ/m^3) caused up to 20% PPO activity decrease (Figure 2.1), while 90% inactivation was obtained only by providing the highest energy density (i.e. 1476 MJ/m^3), corresponding to 45 min of treatment. These results are in agreement with those reported in the literature for PPO inactivation in apple and pineapple juices by means of US processing (Costa et al., 2013; Abid et al., 2014b; Silva et al., 2015). Enzyme inactivation caused by US treatment has been attributed to different mechanisms, including acoustic cavitation, which is responsible for extreme localized increase of pressure and temperature, and propagation of acoustic shock waves, that result in strong shear stresses, promoting protein denaturation (Feng, Yang, & Hielscher, 2008; Mawson et al., 2011). It is worth to note that US treatment was more effective in decreasing PPO activity than heat only (TT_c), i.e. simulating the temperature increase obtained during US without sonication (Figure 2.1). These results are in agreement with those by Sulaiman et al. (2015), who reported that 10 min US at 32 °C was equivalent in terms of PPO inactivation to thermal treatment at 65 °C. These data indicate an acoustic effect on PPO activity clearly distinguishable from the thermal one, although in our experimental conditions the temperature approached 40 °C (Table 2.1).

Overall, data confirmed that HPH and US treatments are scarcely efficient in inactivating PPO, unless high energy density values are provided by applying a high number of passes of sample in the homogenization valve or long US times. These conditions, however, do not meet industry requirements for food processing at low energy input. Therefore, in this paper the effects of combined HPH and ultrasonication with (HPH-US) and without (HPH-US-TT) temperature control were studied and compared in respect to the energy density provided to the sample (Figure 2.2). Approximately 60% reduction of PPO activity was obtained by subjecting the apple juice to single pass HPH (150 MPa) followed by 15 min US with temperature control (HPH-US) (642 MJ/m^3, 47 °C). On the contrary, the analogous treatment carried out without temperature control (HPH-US-TT) caused complete enzyme inactivation after 4 min US (362 MJ/m^3, 74 °C). Even more effective was the US-TT treatment. In this case, PPO was totally inactivated by providing the sample 297 MJ/m^3 of energy density, corresponding to 6 min treatment (74 °C). It is noteworthy that, heat only (TT), i.e. generated by providing the sample the same time-temperature combinations received during US without temperature control, was less effective in inactivating the enzyme than in combination with US, in agreement with the literature (Cheng, Zhang, & Adhikari, 2013). These results again confirm that, besides cavitation phenomena, the *in situ* heat, which is generated during US treatment, greatly contributed to enzyme inactivation.

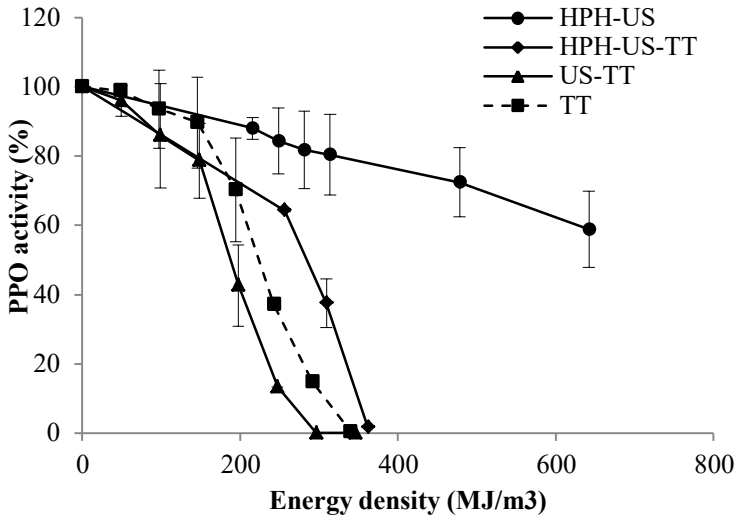


Figure 2.2. PPO activity in apple juice subjected to combined high pressure homogenization (150 MPa) and ultrasound (up to 15 min) with (HPH-US) and without (HPH-US-TT) temperature control, as function of energy density. TT: heat treatment obtained by providing the sample the same time-temperature combinations received during US without temperature control.

The acoustic effect was discriminated from the thermal one by computing the rate constants for PPO inactivation from the slopes of the linear regression ($p<0.05$) of the enzyme activity in apple juice *vs* energy density (Table 2.2). As expected, the estimated rate constants for PPO in apple juice subjected to HPH, US and HPH-US were lower than those obtained by the treatments without temperature control (i.e., US-TT and HPH-US-TT). Only slight differences were observed between US provided alone and in combination with HPH, regardless the temperature regime. This result would confirm the negligible HPH contribution to enzyme inactivation. Moreover, the estimated inactivation rate constants for PPO in apple juice subjected to US and US-TT were greater than those computed for samples subjected to heat only (TT_c and TT, respectively). Therefore, different contributions to enzyme inactivation can be suggested for US and heat.

Table 2.2. Rate constants of PPO activity in apple juice subjected to high pressure homogenization (HPH), ultrasound with (US) or without (US-TT) temperature control, and combinations of high pressure homogenization and ultrasound with (HPH-US) or without (HPH-US-TT) temperature control, *vs* energy density (MJ/m^3), and correspondent determination coefficients. Data on heat provided according to the same time-temperature combinations received during US with (TT_c) or without (TT) temperature control are also shown.

Treatment	k (% m^3/MJ) ± standard error	R^2
HPH	0.024 ± 0.004	0.814
US	0.067 ± 0.009	0.922
HPH-US	0.067 ± 0.007	0.883
TT_c	0.049 ± 0.003	0.974
US-TT	0.372 ± 0.053	0.787
HPH-US-TT	0.252 ± 0.041	0.820
TT	0.360 ± 0.042	0.802

To actually differentiate cavitation and heat impacts to PPO inactivation, the acoustic and thermal contributions were estimated. The acoustic contribution to PPO inactivation was estimated to account for approximately 18%; the thermal one explained for 73% and 97% the treatments performed under controlled and uncontrolled temperature regimes, respectively. These results indicate that heat and US have synergistic effect on PPO inactivation when carried out under controlled temperature regime, being the remaining unaccounted contribution equal to 9%. On the contrary, the US *in situ* generated heat was the main contributor to enzyme inactivation of the treatments performed without temperature control. In this case, shorter treatment times were required to achieve inactivation. A synergistic inactivation of PPO was also found by Cheng et al. (2013) during thermosonication at temperatures not exceeding 55 °C, whilst the extent of synergism decreased above this temperature.

Overall, these data showed that HPH treatment was scarcely effective in inactivating PPO in apple juice even in combination with US. Thus, HPH does not represent a

suitable technology for PPO inactivation in apple juice. On the contrary, US, especially when provided without temperature control, allowed PPO total inactivation to be achieved at energy densities and process time likely compatible with industrial needs.
To gain an insight into the mechanism responsible for PPO inactivation during US treatment, SDS-PAGE analysis was performed. Due to the very low recovery of PPO extracted from apple juice (data not shown), a mushroom tyrosinase model system in phosphate buffer was considered, having specific activity comparable to that of PPO in apple juice. The model system was subjected to 45 min US as well as 7 min TT, providing energy densities of 1479 and 346 MJ/m^3, respectively. These conditions were selected because they allowed to achieve complete PPO inactivation in apple juice. Results showed that sonication and heat inactivation effects on model PPO were comparable to those found for the PPO in apple juice. In particular, the US and TT caused 90% and total enzyme inactivation, respectively (data not shown). Figure 2.3 shows the results obtained by SDS-PAGE for US and TT treated, and untreated PPO model solution. Untreated sample showed clear bands of about 45 kDa and 13 kDa corresponding to H and L subunits of the PPO tetramer (Ismaya et al., 2011). It can be noted that the bands intensity of the US and TT treated samples was lower than that of the control sample, probably due to protein loss during the ultrafiltration step. Nevertheless, the SDS-PAGE analysis showed no differences in the electrophoretic pattern for mushroom PPO subjected to either heating or US, thus excluding the breakdown of the H and L subunit fragments. These results are consistent with those found in the literature. FTIR spectra analysis indicated that PPO was completely inactivated at 70 °C due to irreversible change in the enzyme structure (Baltacioğlu et al., 2015). Overall, these data suggest that heating and ultrasonication are both responsible for changes in the tertiary structure without causing fragmentation of the enzyme subunits.

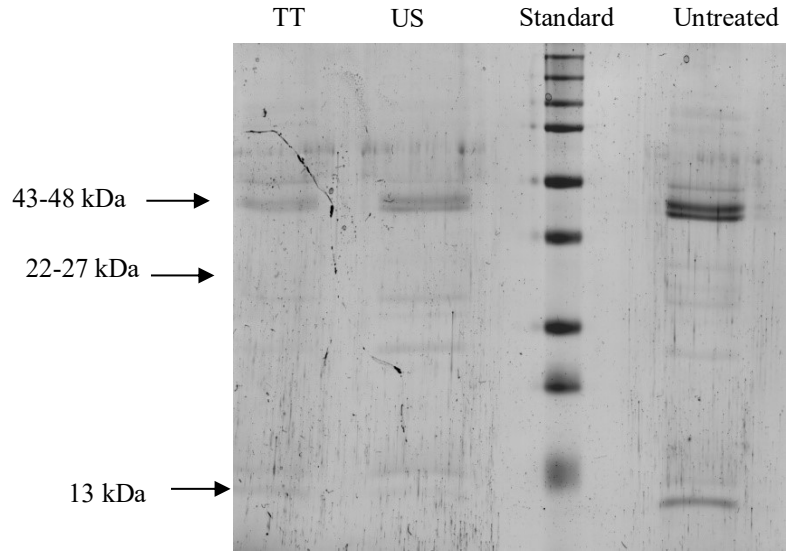


Figure 2.3. SDS-PAGE separation patterns of thermally treated (TT) (346 MJ/m^3), ultrasonically treated (US) (1476 MJ/m^3) and untreated (Untreated) mushroom PPO model solution. Molecular weights of a molecular standard (Standard) are reported in C.

2.4. Conclusions

Acquired results confirmed that HPH treatment was scarcely effective in affecting PPO activity. Only 50% PPO inactivation was achieved at energy densities of about 1500 and 640 MJ/m^3 by applying HPH individually or in combination with US. On the contrary, US allowed a greater inactivation (around 80%) to be obtained at energy densities higher than 900 MJ/m^3. US process without temperature control at lower energy density (about 300 MJ/m^3) and for shorter treatment time (5-6 min) caused total PPO inactivation. Results clearly indicated that US *in situ* generated heat greatly contributed for more efficient enzyme inactivation. In light of this, US process performed under uncontrolled temperature regime might be an alternative technology for enzymatic inactivation in fruit derivatives. In fact, instead of increasing ultrasound energy input and dissipate the heat produced during the treatment, apply low energy densities and exploit the *in situ* generated thermal effect would be a feasible way to inactivate PPO.

Chapter 3

Effect of HPH and low frequency US treatments on some physical properties of tomato juices with different concentration levels

HPH and US are nowadays proposed as novel techniques to steer desirable structure and functionality of fruit and vegetable derivatives. In particular, changes in the physical properties of biopolymers, such as cellulose, starch, pectin and protein, have been described in fruits and vegetables (e.g. banana, mango, pineapple, peach, tomato, broccoli, carrot) derivatives subjected to HPH and low frequency US treatments (Bengtsson & Tornberg, 2011; Calligaris et al., 2012; Kubo et al., 2013; Lopez-Sanchez et al., 2011a; Lopez-Sanchez et al., 2011b; Rojas et al., 2016; Silva et al., 2010). Modification of biopolymers physical properties are reported to highly depend on matrix characteristics and HPH and US intensity, that is pressure level and number of passes applied during HPH, or ultrasonication time (Anese et al., 2013; Augusto et al., 2013; Lopez-Sanchez et al., 2011b; Tan and Kerr, 2015; Vercet et al 2002). From an industrial point of view, the choice between HPH and US to steer plant food material physical properties goes through the evaluation of advantages and drawbacks of each technology. Final product characteristics as well as energy and ownership costs would represents the driving criteria. The energy exchanges involved during HPH and US processes are represented by the energy density, which is the amount of energy provided to the fluid per unit volume during the process, as well as the power demand and energy consumption of the equipment (Raso et al., 1999). To our knowledge, very few data are available in the literature about HPH and US energy aspects (Baumann, Martin & Feng, 2005; Bermudez-Aguirre & Barbosa-Cánovas, 2012; Donsì et al., 2013; Mañas et al., 2000; Stang, Schshmann, Schubert, 2001).

It has been already demonstrated that both HPH and US might modify tomato physical properties (Anese et al., 2013; Colle et al., 2010b; Kubo et al., 2013; Panozzo et al., 2013; Tan & Kerr, 2015). However, to our knowledge, HPH and US performances in the attempt to deliver functionality of plant-based foods are hardly comparable due to scarce information. Moreover, data on the role of tomato solids concentration in affecting changes in physical properties as induced by HPH or US are fragmentary (Bayond et al., 2008; Bayod & Tornberg, 2011; Valencia et al., 2003).

3.1. Aim of the study

The aim of this study was to investigate the use of HPH and US for modifying some physical properties of tomato juice thus obtaining derivatives with improved technological characteristics, especially structure and rheological properties. To this purpose, tomato juices with different soluble solids contents were subjected to HPH and US for increasing pressure levels or treatment time periods, respectively, and the changes in some physical properties were studied. Finally, to steer industry choice on the best performing process, estimation of the energy density transferred to the juice during processing, as well as measurement of electrical energy consumption of the laboratory

devices were performed to compare HPH and US processes from the point of view of energy efficiency.

3.2. Material and Methods

3.2.1. Sample preparation

Tomato juice at 5.0, 7.5 and 10.0 °Brix (corresponding to 5.2±0.1, 8.3±0.1 and 10.7±0.1% dry matter, respectively) was obtained by dilution of commercial tomato paste (21 °Brix) in distilled water. The pH of the juice was 4.5±0.1.

3.2.2. Treatments

3.2.2.1. HPH

Samples were subjected to HPH treatment using the same equipment reported in paragraph 2.2.3.1 (pag. 22).In particular, the first valve was the actual homogenization stage and was set at increasing pressures from 20 to 150 MPa. The second valve was set at the constant value of 5 MPa. Aliquots of tomato juice were introduced into the equipment at 10±1 °C and cooled using a water-ice ice bath just after the treatment. The maximum temperature reached by the sample was 45±2 °C.

3.2.2.2. Low frequency US

Samples were subjected to US using the same equipment reported in paragraph 2.2.3.2 (pag. 22). Aliquots of 150 mL of tomato juice were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass vessel. The tip of the sonicator horn was placed in the centre of the solution, with an immersion depth in the fluid of 25 mm. Treatments were carried out for increasing time periods, up to 30 min. During the treatments, the temperature was controlled using a cryostat set at 4 °C to dissipate the heat generated during treatment. Temperature never exceeded 45±2 °C. Following the treatments, the samples were cooled in a water-ice ice bath.

3.2.3. Energy density computation

The energy density (E_v, MJ/m^3) transferred from the homogenization valve to the sample was determined by using the same procedure described in paragraph 2.2.5 according to eq. 2.1 (pag. 23)

The power density (P_v, kW/m^3) transferred from the ultrasound probe to the sample was determined by using the same procedure described in paragraph 2.2.5, eq. 2.2 (pag.23).

The energy density (E_v, MJ/m^3) transferred from the ultrasound probe to the sample was determined by using the same procedure described in paragraph 2.2.5, eq. 2.3 (pag. 23).

3.2.4. Electrical energy consumption measurement

For both the HPH and US treatments the energy requirement was estimated by measuring the electrical consumption at the mains supply. The high pressure homogenizer was supplied with three-phase 400 V electrical power. Thus a three-phase energy logger was inserted (Kilo Box, Electrex, Reggio Emilia, Italy) to measure the electrical consumption (MJ/m^3) as active power, that is the effective power used by the apparatus, and the power factor, that is the ratio between the “active power” and the

"apparent power" related to the power supplied by the net. The power factor is in the range 0-1 and it should be as high as possible for optimal exploitation of the electrical energy supplied. The ultrasonic processor was instead supplied with single-phase 230 V electrical power, and a power meter (PC-300, Lafayette, Taiwan) was connected to measure the electrical power and thus calculate the electrical energy (MJ/m^3) for the whole treatment.

3.2.5. Analytical determinations

3.2.5.1. Soluble solids (°Brix)

The soluble solids (°Brix) were measured using a hand Refractometer (Unirefrax, S.A Bertuzzi, Milan, Italy). Measurements were performed at 25 °C. The refractometer prism was cleaned with distilled water before each analysis.

3.2.5.2. Temperature measurement

The sample temperature was measured just before and immediately after (i.e. before the cooling step) each treatment by a copper-constantan thermocouple probe (Ellab, Hillerød, Denmark) immersed in the tomato juice, connected to a portable data logger (mod. 502A1, Tersid, Milan, Italy).

3.2.5.3. Colour

Colour analysis was carried out using a tristimulus colorimeter equipped with a CR-300 measuring head (Chromameter-2 Reflectance, Minolta, Osaka, Japan). The instrument was standardised against a white tile before measurements. Colour was expressed in L*, a* and b* scale parameters and a* and b* were used to compute the hue angle ($\tan^{-1}$ b*/a*). An increase of this colour parameter was used as an index of redness loss.

3.2.5.4. Rheological properties

Rheological measurements were carried out using a controlled stress rheometer (SR5, Rheometric Scientific, Germany) equipped with serrated parallel plate geometry (40 mm diameter, 2 mm gap). The temperature was maintained constant at 25 °C using a Peltier system. Samples were placed between the plates of the rheometer and left to rest 5 min after loading before testing. This resting time was sufficient for the sample to relax and reach a constant temperature. Dynamic strain sweep tests were carried out at 1 Hz between 0.1% and 100% strain to determine the linear viscoelastic range. Frequency sweep tests were performed from 0.1 to 10 Hz within the linear viscoelastic range. Data obtained were storage modulus (G'), loss modulus (G") and tan δ (G"/G'). Statistical comparisons were made at 0.1 Hz.

3.2.5.5. Bostwick flow index

Samples were placed into Bostwick consistometer (RG Strumenti srl, Parma, Italy). This empirical test consists in allowing the sample to flow under its own weight along a sloped stainless steel tray for 30 s at room temperature (22 °C). The distance (cm) covered by the sample was recorded and the inverse of the distance (cm^{-1}) was used to

express the Bostwick consistency index. An increase of this parameter is associated to high sample consistency.

3.2.5.6. Precipitate weight ratio

Precipitate weight ratio was determined using the method of Colle et al. (2010a), with minor modifications. Tomato juice (25 g) was centrifuged (Beckman, Avant J-25 centrifuge, Palo Alto, California, USA) at 45000 *g* for 30 min at 15 °C. The percentage of precipitate weight ratio of the pellet was calculated as:

$$P = (W_p/W_t) \cdot 100 \qquad \text{(eq. 3.1)}$$

where W_p and W_t are the precipitate and tomato juice weights, respectively.

3.2.5.7. *Determination of degree of esterification*

The determination of the degree of esterification was carried out using the method of Chou and Kokini (1987). 60 g of tomato juice were centrifuged (Beckman, Avant J-25 centrifuge, Palo Alto, California, USA) at 7500 *g* for 15 min at 20 °C. The supernatant was filtered under vacuum through filter paper (RPE ACS, Carlo Erba, Milano, Italy) and an equal volume of 2-propanol was added to the filtrate to precipitate the isopropanol-insoluble pectins. After 15 min stirring, the suspended solids in the water-isopropanol mixture were centrifuged at 7500 *g* for 15 min at 20 °C and isopropanol was removed by means of vacuum dehydration (Laborota 4001 Efficient, Hedolph Instruments, Schwabach, Germany). The water-soluble pectins were decoloured by acetone:pentane solution (2:1). 10 mL of 1% decoloured pectin solution were titrated with 0.05 N NaOH (titration A). Afterwards, 20 mL 0.5 N NaOH were added to de-esterify the pectin and, after 30 min, 20 mL 0.5 N HCl were added to neutralise the NaOH. This mixture was titrated with 0.1 N NaOH (titration B), using phenolphthalein as indicator. The degree of esterification (DE), expressed as a percentage, was calculated using the following equation:

$$DE = [B/(A+B) \cdot 100] \quad \text{(eq.3.2)}$$

3.2.5.8. Images

Tomato juice images were captured using a digital camera (Nikon D3, Nikon Corporation, Tokyo, Japan) mounted on an adjustable stand positioned 50 cm above a black cardboard base where the sample was placed. Light was provided by two 250 W frosted photographic floodlights in a position allowing minimum shadow and glare. Images were saved in the jpg file format.

3.2.5.9. Light microscopy

Light micrographs were taken using a Leica DM microscope (Leica DM 2000, Leica Microsystems, Heerburg, Switzerland) connected with a Leica EC3 digital camera (Leica Microsystems, Heerburg, Switzerland). The samples were analysed with 20x lens.

The images were acquired and processed using the Leica Suite LAS EZ (Leica Microsystems Heerburg, Switzerland) software application. Images were saved in jpeg format resulting in 2048 x 1536 pixels. Tomato juice microstructure was analysed by using an optical microscope (Leica DM 2000, Leica Microsystems, Heerburg, Switzerland). The pictures were taken by a digital camera (Leica EC3, Leica Microsystems, Heerburg, Switzerland), using the Leica Suite LAS EZ software (Leica Microsystems, Heerburg, Switzerland).

3.2.6. Data analysis

The results are the average of at least two measurements carried out on two replicated experiments ($n \geq 4$). Data are reported as mean value ± standard error. Statistical analysis was performed using R v.2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p < 0.05$).

3.3. Results and discussion

3.3.1 Effect of HPH and US processing on tomato juice physical properties

Tomato juices with 5.0, 7.5 and 10.0 °Brix were subjected to HPH for increasing pressures up to 150 MPa or US for increasing time periods up to 30 min. Figure 1 shows the macroscopic images of 7.5 °Brix untreated as well as 150 MPa HPH and 30 min US treated tomato juices. Differences in tomato appearance, mainly relevant to color and consistency, can be observed between the untreated and treated samples.

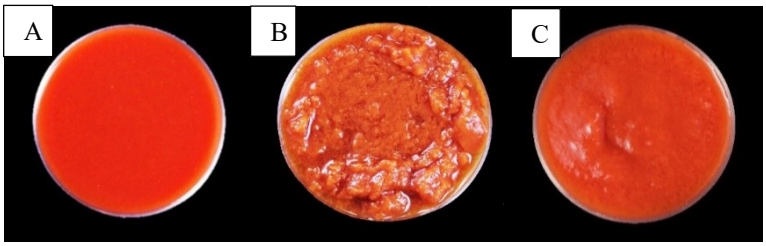


Figure 3.1. Images of 7.5 °Brix untreated (A), 150 MPa HPH (B) and 30 min US (C) treated tomato juices.

To quantify these changes, samples were analyzed for their rheological properties, color, precipitate weight ratio, pectin esterification degree and cell integrity. Unprocessed and HPH or US processed tomato juices were undergone to dynamic, small deformation tests to acquire information on structure. Both storage (G') and loss (G'') moduli were frequency dependent and a prevalence of G' over G'' was found, indicating a weak gel-like behaviour of tomato juice (data not shown). The storage modulus and tan δ at a constant frequency (0.1 Hz) were used to compare samples subjected to HPH and US processing (Figure 3.2). As a rule, the higher G' and the lower tan δ the more elastic and solid-like the material. G' and tan δ values of HPH and US treated tomato juices were respectively always greater and lower than those of the untreated samples ($p < 0.05$). This

suggests a higher number of elastic interactions in processed tomato juices, which resulted in a stronger structure. Results are in agreement with those reported by other authors on the influence of HPH and US on viscoelastic properties of tomato derivatives (Anese et al., 2013; Lopez-Sanchez et al., 2011a and b; Vercet et al., 2002; Wu et al., 2008). It is noteworthy that Tan and Kerr (2015) found opposite results with a decrease in rheological parameters upon HPH treatment. Such controversial data can be attributed to differences in homogenization devices used as well tomato juice initial properties (e.g. solid content and particle size) and technological history. In our experimental conditions the extent of changes in viscoelastic properties increased with the increase in juice concentration. In particular, a significant G' increase was found for the 5.0 and 7.5 °Brix tomato juices subjected to HPH up to 50 MPa, while no further increase in storage modulus was observed at higher pressures, except for the 5.0 °Brix tomato sample. In the latter case, the 150 MPa treatment caused a significant decrease in G' values. It is likely that the inter-particle interactions would be less favored due to the high dilution degree of the system, in agreement with Tan and Kerr (2015).

By contrast, G' of the 10.0 °Brix tomato juice increased progressively with the increase of pressure, reaching at 150 MPa 4 times higher values than the untreated sample, in agreement with literature (Augusto et al., 2013). Similarly, the storage modulus and tan δ of the 5 min US treated tomato juices, at all concentrations, were respectively higher and lower than those of the untreated samples ($p>0.05$). No significant changes in the viscoelastic properties were observed among samples subjected to increasing US times ($p>0.05$).

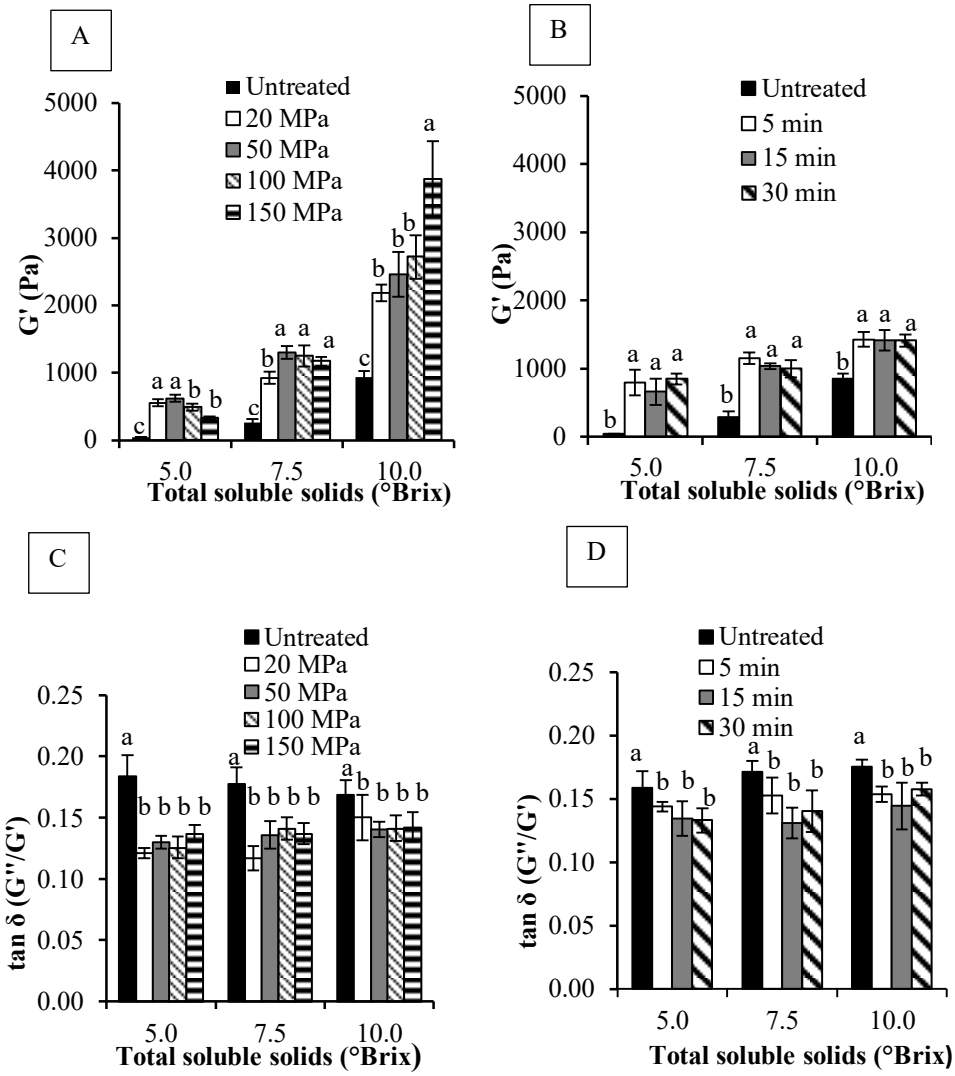


Figure 3.2. Storage modulus (G') and tan δ at 0.1 Hz of 5.0, 7.5 and 10.0 °Brix tomato juices subjected to high pressure homogenization (HPH) (A, C) and ultrasound (US) (B, D) processes.

Tomato juice consistency was also evaluated by Bostwick consistometer, which is a widely used tool for quality control at the industrial level. HPH and US induced a significant increase ($p<0.05$) in juice consistency, in agreement with the data relevant to the storage modulus (Figure 3.3). Results suggest that both HPH and US were responsible for modifications in the physical properties of tomato samples, which are attributable to cell rupture. As shown in Figure 3.4 HPH caused a progressive cell disruption. In particular, microscopy analysis of treated samples showed that the percentage of intact cells present in 16 mm^2 of sample were 27% and 7% in the 20 MPa

and 50 MPa treated juices, respectively. No intact cells were found upon 100 MPa and 150 MPa treatments. 80%, 53% and 27% undamaged cells were still present in the 5, 15 and 30 min US treated samples, respectively.

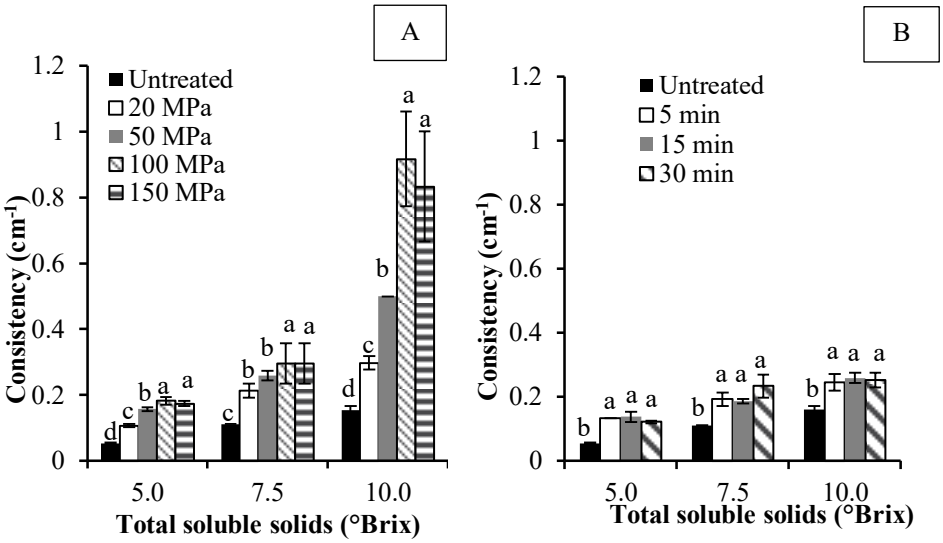


Figure 3.3. Bostwick consistency of 5.0, 7.5 and 10.0 °Brix tomato juices subjected to high pressure homogenization (HPH) (A) and ultrasound (US) (B) process.

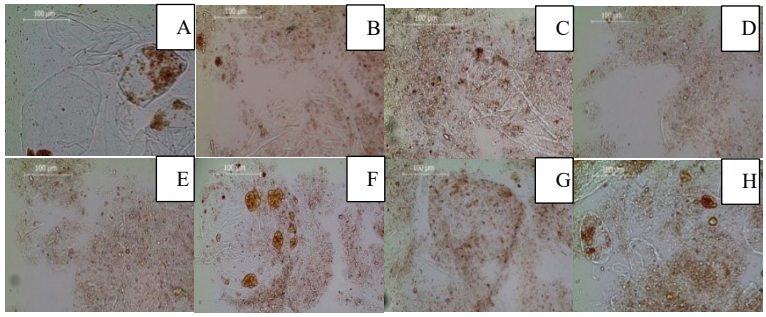


Figure 3.4. Micrographs of 5.0 °Brix untreated (A), and HPH (B: 20 MPa, C: 50 MPa, D: 100 MPa, E:150 MPa) and US (F: 5 min, G: 15 min, H: 30 min) processed tomato juices.

As a consequence of cell rupture, the surface area of the suspended particles increased and tomato constituents were released in the medium. In these conditions, novel inter-particle interactions could have been favoured. These events were accompanied by sample colour bleaching (Table 3.1). By increasing the HPH pressure, the color fading progressively increased in agreement with Kubo et al. (2013). No significant changes in color were found among untreated and US treated samples, except for the 5.0 °Brix one. In this case, the hue angle significantly increased after 5 min treatment, while no further color changes were found by increasing the process time. No differences in colour bleaching were found among samples with different solids content ($p>0.05$). It can be inferred that HPH and US by disrupting cell membrane caused carotenoids dispersion in the medium, where they underwent to isomerization and oxidation being not more protected by cell oxidation being not more protected by cell integrity (Colle et al., 2010b).

Table 3.1. Hue angle (arctan b*/a*) of 5.0, 7.5, 10.0 °Brix tomato juices subjected to high pressure homogenization (HPH) and ultrasound (US) processes. Data relevant to untreated samples are also shown.

Total soluble solids content (°Brix)	Untreated	HPH				US		
		Pressure (MPa)				Time (min)		
		20	50	100	150	5	15	30
5.0	21^{e}	24^{d}	27^{c}	29^{b}	30^{a}	25^{d}	24^{d}	24^{d}
7.5	23^{c}	27^{b}	29^{a}	29^{a}	29^{a}	23^{c}	24^{c}	23^{c}
10.0	22^{d}	23^{cd}	24^{b}	26^{a}	26^{a}	23^{cd}	22^{cd}	22^{cd}

$^{a, b, c, d, e}$: means with different letters in the same row are significantly different ($p<0.05$)
standard error<1

To support this hypothesis, the precipitate weight ratio of untreated and treated tomato juices was determined (Table 3.2). This measure provides an indication of the capacity of the matrix to hold water in the macromolecular network (Colle et al., 2010b). The higher the precipitate weight ratio the stronger the network and its water holding capacity. Specifically, increases from 11% to 22% and from 14% to 36% were found for 5.0 and 7.5 °Brix HPH processed tomato juices, respectively. A greater increase up to 46% in the precipitate weight ratio was obtained for the 10.0 °Brix sample subjected to HPH for increasing pressures. Similarly, US treatments caused significant changes of this indicator compared to the untreated samples in all the tomato juices considered. Treatment times higher than 5 min did not induce further changes of precipitate weight ratio ($p>0.05$), in agreement with the other indexes previously described for the US processed samples.

Table 3.2. Precipitate weight ratio (%) of 5.0, 7.5, 10.0 °Brix tomato juice subjected to high pressure homogenization (HPH) and ultrasound (US) treatments. Data relevant to untreated samples are also shown.

Total soluble solids content (°Brix)	Untreated	HPH				US		
		Pressure (MPa)				Time (min)		
		20	50	100	150	5	15	30
5.0	11±2^{e}	14±0de	18±1^{bc}	19±2^{ab}	22±1^{a}	14±0de	15±0^{cd}	17±1^{bd}
7.5	14±1^{g}	23±1^{d}	27±1^{c}	30±2^{b}	36±1^{a}	20±0^{ef}	22±1de	19±1^{f}
10.0	19±0^{f}	29±1^{d}	37±1^{c}	42±3^{b}	46±2^{a}	23±0^{ef}	23±1^{e}	22±0^{ef}

$^{a, b, c, d, e, f}$: means with different letters in the same row are significantly different ($p<0.05$)

Measurement of the pectin esterification degree of the HPH or US processed tomato juices showed that this parameter did not change in comparison with that of the untreated samples, regardless the tomato juice concentration and intensity of process applied (data not shown). This result is in agreement with the literature in the framework of the effect of HPH and US on pectin molecule (Shpigelman et al., 2015; Anese et al., 2013). Thus, the modifications of colour, rheological properties and water holding capacity of tomato juice upon HPH and US treatments can be mainly attributed to physical events than to chemical ones.

Discrepancies in the observed physical properties between samples subjected to HPH and US are likely attributable to differences in modalities of force transmission during processing and thus in mechanical stresses generated by the two technologies. Considering the 5.0 and 7.5 °Brix tomato juices, similar structure modifications were obtained by applying HPH or US, regardless the process intensity. By subjecting 10.0 °Brix tomato juice to HPH processing, a 2- to 4-fold increase in the viscoelastic properties was observed. On the contrary, approximately 1.5-fold increase in G' was found for the 10.0 °Brix US treated sample, regardless the treatment time. The changes in physical properties of the HPH processed tomato juice suggests that higher particle-particle interactions took place leading to the formation of a stronger network. It can be inferred that the crowding in the homogenization valve increased by increasing the number of suspended material in the sample, thus favouring interactions and, consequently, inducing an increase in consistency as well as gel-like properties. In these conditions the magnitude of mechanical stresses acting on the particles during the flow through the valve could have become much higher, thus increasing the extent of cell disruption. Our findings highlight that stress magnitude in combination with product concentration is crucial for structure development using HPH. Differently, the particle number increase is expected to reduce the efficacy of ultrasonication, because the high initial product consistency could hinder wave propagation (Earnshaw, 1998).

3.3.2 Energy density and electrical energy consumption of HPH and US lab equipment

To estimate HPH and US processes efficiency the energy density and electrical energy consumption were evaluated. Table 3.3 shows the power density measured at ambient temperature at the beginning of the US treatment, as well as the energy density values for both processes. It appears that the energy density values for the US process were higher than those for the HPH treatment, even if the former are possibly underestimated because of their calculation procedure.

Table 3.3. Energy density values generated during HPH and US of 5.0, 7.5 and 10.0 °Brix tomato juices, and power density at ambient temperature relevant to the US treatment.

Total soluble solids content (°Brix)	HPH			US	
	Energy density (MJ/m^3)			Power density (kW/m^3)	Energy density (MJ/m^3)
	50 MPa	100 MPa	150 MPa		
5.0	50	100	150	533	612
7.5	50	100	150	916	635
10.0	50	100	150	1316	659

Values of the electrical energy consumption for HPH and US devices are summarised in Table 3.4. As far as energy consumption of HPH is concerned, the electrical energy values were calculated considering that the lab-scale high pressure homogenizer was capable to treat 2.5 cm^3/s of product. An almost linear correlation between pressure and both electrical energy and power factor was found. Moreover, sample concentration did not affect these parameters. This result was also confirmed by treating deionised water in the high pressure homogenizer. Energy consumption of the US treatment was estimated by integrating the measurements of the instantaneous electric power supplied during a 30 min treatment. US energy consumption showed to be affected by a ± 5 % variability in the measurements, possibly due to a self-regulation of the device itself, which was out of control. This prevented from drawing definite conclusions on the effect of concentration, but did not affect significantly the comparison between treatments. According to Table 3.4, at laboratory scale the high pressure homogenizer presented quite lower energy consumption than the ultrasound device, even if the HPH apparatus gave rise to significantly low power factors which call for correction. This result is consistent with the values of energy density supplied to the tomato juice by the two treatments (Table 3.3).

Table 3.4. Electrical energy consumption of the HPH and US lab devices during tomato juice processing.

Total soluble solids content (°Brix)	HPH						US
	Electrical energy (MJ/m^3)			Power factor			Electrical energy (MJ/m^3)
	50 MPa	100 MPa	150 MPa	50 MPa	100 MPa	150 MPa	
5.0	317	480	644	0.29	0.41	0.51	1369
7.5	310	464	648	0.28	0.40	0.52	1171
10.0	312	462	645	0.28	0.40	0.51	1250

3.4 Conclusions

Results of this study highlighted the influence of stress type and food solids concentration on the changes in tomato juice physical properties induced by HPH and US processing. HPH and US processing were responsible for higher G' and consistency of tomato juices than untreated samples. In particular, such increases were comparable for the 5.0 and 7.5 °Brix treated HPH and US samples. By contrast, increasing tomato juice concentration to 10.0 °Brix, HPH treatments were more effective than US in changing sample rheological properties. These changes were attributed to cell disruption and consequent increase of inter-particle interactions. From an industrial feasibility perspective, the possible application of HPH or US treatments could be interesting for products such as tomato ketchup, juices or sauces. Thus, the acquired results provide information useful to select the most appropriate process to steer physical properties of tomato derivatives. For tomato juices with concentration equal or lower than 7.5 °Brix, the choice between HPH and US should not be performed on the basis of the induced structure modifications because both technologies led to comparable effects. In this context, energy efficiency would drive the choice. Despite the study was performed on lab-scale equipment, the estimated energy density transferred to the juice during processing and the equipment electrical energy consumption here reported can be used to compare HPH and US processes. On the contrary, for tomato juices with higher concentration (10.0 °Brix), HPH treatments resulted very effective in changing sample consistency and gel-like properties, in an extent that was not achievable by applying the US process. Thus, the criteria for technology selection should be based on a product perspective rather than on process costs.

Chapter 4

Effect of low frequency US treatments on microbial decontamination in wastewater from fresh-cut lettuce washing

The effect of low frequency US treatments only or in combination with heating on microbial decontamination has been largely investigated. However, very few studies investigated ultrasound effectiveness for water decontamination deriving from fresh-cut vegetable production (Elizaquível et al., 2012; Gómez-López et al., 2014; Gómez-López et al., 2015).

Fresh-cut industry production requires intensive use of water to both wash and move vegetables along the production line. In order to secure water supply and protect the environment from the adverse effects of the wastewater discharges (EEC 1991), water recycling in the fresh-cut industry has to be improved. Recycling of water that is intended to re-enter the washing step, implies wastewater disinfection. As well known, a 5 Log reduction of pathogenic bacteria is the generally accepted requirement for safe water disinfection. Wastewater decontamination may be accomplished by means of chemical and physical interventions (Casani, Rouhany, & Knøchel, 2005; Olmez & Kretzschmar, 2009). Among these, sodium hypochlorite is the most used due to its low cost and easy use (Olmez & Kretzschmar, 2009; Gil et al., 2009). However, not only wastewater containing chlorine has a great environmental impact, but also chlorination disinfection by-products are known to represent a potential risk for human health (Itoh et al., 2011). Consequently, there is great effort to find suitable technologies to allow wastewater recycling (Artés et al., 2009; Casani et al., 2005; Olmez & Kretzschmar, 2009). Low frequency ultrasounds have been suggested as a technology alternative to chlorination for wastewater decontamination (Neis & Blume, 2002; Piyasena, Mohareb, & McKellar, 2003).

4.1 Aim of the study

In this study, the efficacy of low frequency ultrasound in decontaminating wastewater deriving from fresh-cut vegetable washing was investigated. To this aim, wastewater obtained by washing fresh-cut lamb's lettuce was subjected to low frequency ultrasounds, provided in pulsed or continuous modality, with or without temperature control. The decontamination efficacy of the treatments was evaluated on both the native microflora and inoculated pathogenic bacteria, i.e. *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica*. These microorganisms were chosen due to their natural occurrence in a water environment and because they are generally considered indicators of fecal contamination (Szewzyk et al., 2000). The final goal was to find the potentiality of combined ultrasound with *in situ* generated heat in the attempt to implement strategies for efficient management of water resource in the fresh-cut industry. To this regard, the decontamination efficacy was related to the ultrasound cavitation and heat contributions.

4.2 Materials and methods

4.2.1Preparation of fresh-cut vegetable wash water

Lamb's lettuce (*Valerianella locusta* Laterr.) was purchased from a local market. Lettuce leaves were placed into a beaker containing tap water at 18 ± 2 °C (the vegetable-water ratio was 1:30 w/v). After 1 min of washing, water was separated from the leaves by using a domestic salad spinner.

4.2.2 Bacterial strains and inoculum preparation

The microorganisms used for inoculum were *Listeria monocytogenes Escherichia coli* and *Salmonella enterica* subsp. *enterica* 9898 DSMZ, obtained from the bacterial culture collection of the Department of Food Science of the University of Udine (Italy). Strains were maintained at -80 °C in Brain Heart Infusion broth (BHI, Oxoid, UK) with 30% sterile glycerol as cryoprotectant until use. Strains were incubated in BHI at 37 °C for 24 h, subsequently cultured in 5 mL of BHI at 37 °C for 24 h, and finally collected by centrifugation at 14170 *g* for 10 min at 4 °C (Beckman, Avanti TM J-25, Palo Alto, CA, USA) and washed three times with *Maximum Recovery Diluent (*MRD, Oxoid, UK). The final pellets were suspended in MRD and used as inoculum. A final concentration of approximately 10^6 CFU/mL was obtained for each bacteria suspension.

4.2.3 Low frequency US treatments

Samples were subjected to US using the same equipment reported in paragraph 2.2.3.2 (pag. 22). Aliquots of 200 mL of wash water inoculated or not with *L. monocytogenes*, *E. coli* and *S. enterica* were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass vessel. The tip of the sonicator horn was placed in the centre of the solution, with an immersion depth in the fluid of 10 mm. The ultrasound treatments were performed for increasing lengths of time up to 20 min. During the ultrasonication experiment, the temperature was either controlled (<35 °C) using an ice bath, to dissipate the heat generated during treatment, or uncontrolled, leaving the temperature to rise due to heat dissipation. The sonicator operated either in pulsed mode or continuous mode. In the pulsed mode, the pulse duration period of 0.5 s was followed by a pulse interval period of 0.5 s, during which the sonochemical reactor was switched off. Before and after each experiment, the ultrasound probe was disinfected by washing with ethanol followed by through rinsing with sterile water.

4.2.4 Thermal treatment

The total temperature-time combination received by water during continuous ultrasound under uncontrolled temperature regime was applied to the wastewater in the absence of the ultrasound treatment. To this purpose, aliquots of 200 mL of wash water were introduced into 250 mL capacity glass vessel and heated in a thermostatic water bath (Ika Werke, MST BC, Staufen, Germany) under continuous stirring, by mimicking the same temperature rise produced by the probe during continuous ultrasound treatment under the uncontrolled temperature regime.

4.2.5 Microbiological analysis

Both naturally present and inoculated microorganisms were quantified at different time intervals during the ultrasound and heat treatments. The wastewater samples were diluted 10 fold with MRD (*Oxoid*, UK*).* Total viable count of non inoculated water was enumerated by spreading onto plates with Plate Count Agar (PCA, Oxoid, UK) and incubating at 30 °C for 48 h. *L. monocytogenes* and *S. enterica* concentrations were determined by plating on Palcam Agar (PA, Oxoid, UK) and Xylose Lysine Desoxycholate agar (XLD, Oxoid, UK), respectively, at 37 °C for 48 h, while *the C*oli ID medium (BioMerieux, Mercy L'Etoile, France) was used for *E. coli* concentration determination, followed by incubation at 37 °C for 24 h.

Preliminary trials were carried out on the non inoculated wastewater to check for *Salmonella* spp. and *L. monocytogenes* presence and enumerate *E. coli*. For *Salmonella* spp., 25 mL of wastewater was diluted with 225 mL of Buffered Peptone Water (BPW, Oxoid, UK), homogenised in a Stomacher *Lab-Blender 400* (VWR International PBI srl, Milano, Italy) for 2 min and incubated at 37 °C for 24 h. Aliquots of 0.1 mL of BPW were added with 9.9 mL Rappaport Vassiliadis (RV, Oxoid, UK) and incubated at 42-43 °C for 18-24 h. Presence/absence of *Salmonella* spp. was checked by spreading onto XLD agar plates and incubating at 37 °C for 24 h. For *L. monocytogenes*, 25 mL of wastewater were diluted with 225 mL of Fraser Broth (FB, Oxoid, UK), homogenised in a Stomacher for 2 min and incubated at 30 °C for 36-48 h. 1 mL of FB was added with 9 mL of FB and incubated at 37 °C for 24-48 h. Presence/absence of *L. monocytogenes* was checked by spreading onto PA plates and incubating at 37 °C for 24-48 h. To evaluate the presence of *E.coli* the Coli ID medium at 37 °C for 24 h was used.

In order to investigate whether treatments were responsible for bacteria sub-lethal injury, resuscitation trials were carried out. For each inoculated strain, 10 mL of wastewater was transferred into 10 mL of BHI broth and then incubated at 30 °C for 2h. Afterwards, presence/absence of *L. monocytogenes*, *E. coli* and *S. enterica* was checked by spreading onto PA, Coli ID and XLD agar media, respectively.

4.2.6 Temperature measurement

The temperature was recorded as a function of time using a copper-constantan thermocouple probe (Ellab, Denmark), connected to a data-logger (CHY 502A1, Tersid, Milano, Italy).

4.2.7 Power and energy density computation

The power density (P_v, W/L) transferred from the ultrasound probe to the sample was determined by using the same procedure described in paragraph 2.2.5, eq. 2.2 (pag. 23). The energy density (E_v, kJ/L) was estimated by integration of the power density as described in paragraph 2.2.5, eq. 2.3 (pag. 23)

4.2.8. Statistical analysis

The results reported here are the average of at least two measurements carried out on two replicated experiments ($n \geq 4$). Data are reported as mean value ± standard deviation. Statistical analysis was performed using Statistica for Windows (ver. 5.1, Statsoft Inc. Tulsa, USA, 1997). Bartlett's test was used to check the homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p<0.05$). Linear regression analysis was performed by using Microsoft Excel 2007. The goodness of fitting was evaluated based on visual inspection of residual plots and by the calculation of R^2 and p.

4.3 Results and discussion

4.3.1. Decontamination efficiency of continuous ultrasounds provided under controlled temperature regime

Initial total microbial count of wastewater deriving from fresh-cut lamb's lettuce wash water was 4.92 ± 0.15 Log CFU/mL. This value was in the same magnitude range of those reported in the literature for wastewater obtained by washing fresh-cut vegetable (Elizaquivel et al., 2011; Gómez-López et al., 2015). As reported by Ignat et al. (2015) for wastewater obtained from lamb's lettuce washed in analogous conditions as those performed in the present study, the microbial count was mainly represented by *Pseudomonas* spp, *Enterobacteriaceae* and total coliforms. No presence of *L. monocytogenes, E. coli* and *S. enterica* cells was detected in wastewater.

Wastewater obtained by washing fresh-cut lettuce was subjected to ultrasound treatment for up to 20 min in continuous mode and controlled temperature regime. To avoid temperature increase, the vessel containing the sample was placed into an ice bath to remove the heat generated during the ultrasound process into the fluid. Under these conditions, temperature reached 31 °C after 5 min treatment and it set at 34 °C at 10 min. Thus, the controlled temperature regime allowed values never exceeding 35 °C to be obtained. The power density transferred from the ultrasound probe into the fluid, quantified calorimetrically using eq. 2.2, was equal to 270 W/L. Accordingly, the acoustic energy density values ranged between 15 kJ/L and 314 kJ/L (eq. 2.3), depending on treatment time.

Figure 4.1 shows the decontamination efficiency of continuous ultrasound provided under controlled temperature regime against the total microbial count as well as *L. monocytogenes, E. coli* and *S. enterica* inoculated in the wastewater obtained by fresh-cut lettuce washing. Following the ultrasound treatments, Log reductions of the total microbial count as well as *L. monocytogenes, E. coli* and *S. enterica* of the wash water increased linearly with exposure time ($p<0.05$). In particular, the rate constants computed from the slopes of the linear regression of the logarithm of microbial counts as a function of ultrasonication time were 0.127, 0.09, 0.195 and 0.226 min^{-1} ($0.783<R^2<0.973$) for native microflora, *L. monocytogenes, E. coli* and *S. enterica,* respectively. These differences in rate constants indicate different resistances to ultrasonication among the microorganisms. A total microbial count reduction of approximately 2.8 Log units was obtained after 20 min application of this treatment. Based on the above rate constants, a 5 Log reduction of *L. monocytogenes, E. coli* and *S.*

enterica, that is the minimum requirement for water disinfection, can be achieved by the application of 56, 26 and 22 min of ultrasound, respectively. It is noteworthy that these treatments are hardly applicable at the industrial level because time and cost consuming. In our experimental conditions, higher decontamination effects were achieved as compared with those of the literature. Neis and Blume (2002) reported that reductions of 0.9 and 2.9 Log units of fecal streptococci and *E. coli*, respectively, were achieved following 60 min at 400 W/L. Similar Log reductions of total coliforms and fecal streptococci in municipal wastewater subjected to 1500 W/L power density were reported by Drakopoulou et al. (2009). Ayyildiz, Sanik and Ileri (2011) found that *E. coli* Log reductions ranged from approximately 0.5 and 1.1 for municipal wastewater processed at 75 to 300 W/L for 10 min. Elizaquivel et al. (2011) reported 2.4 Log reductions of *E. coli* O157:H7 inoculated in fresh-cut vegetable wastewater following 30 min ultrasonication at 280 W/L, while 60 min were required to achieve complete inactivation (5 Log reductions). Similarly, Gómez-López et al. (2015) reported that 30 min ultrasound treatment at 280 W/L of wastewater obtained by lettuce washing allowed 2 Log reductions for *E. coli* and *S. enterica*, and 1 Log reduction for *L. monocytogenes* to be achieved.

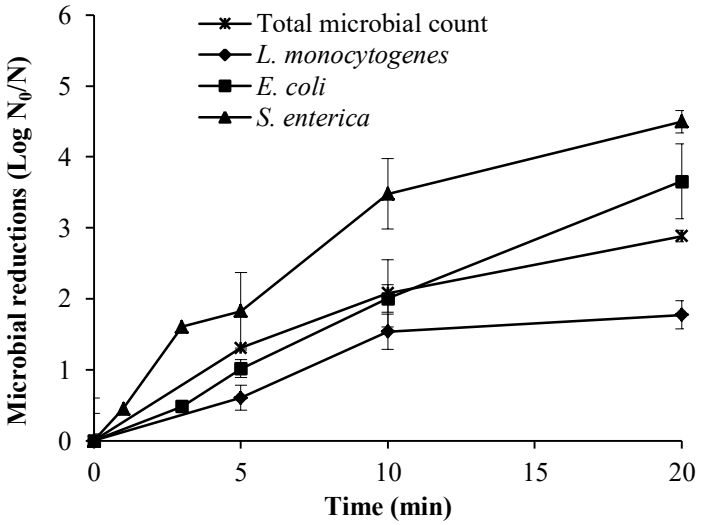


Figure 4.1. Log reductions of total microbial count, *L. monocytogenes*, *E. coli* and *S. enterica* in wastewater obtained by fresh-cut lamb's lettuce washing, subjected to continuous ultrasound under controlled temperature regime.

To actually quantify the effect of ultrasound, the decimal reduction time D_{US} for the inoculated pathogenic bacteria was calculated using procedures analogous to those employed in thermal death time studies. In particular, D_{US} was defined as the ultrasonication time needed to reduce the number of microorganisms by 90% at a given ultrasound power. D_{US} values of 11.1, 5.1 and 4.4 min were obtained for *L. monocytogenes, E. coli* and *S. enterica,* respectively. According to the above mentioned

definition, the higher the D_{US} value, the less the microorganism susceptibility to the ultrasonication power. Therefore, *S. enterica* resulted to be slightly more susceptible to the ultrasound treatment than *E. coli*, that in turn was more sensitive than *L. monocytogenes,* in agreement with Gómez-López et al. (2015). The greater resistance of *L. monocytogenes* to ultrasound treatments can be attributed to its Gram status. As known, the Gram-positive cell wall of microorganisms presents a thicker and more tightly adherent peptidoglycan layer than that of the Gram-negative microorganisms (Cummins, 1989). Thus, *L. monocytogenes* would be capable to better withstand extreme pressure and temperature variations due to cavitation.

4.3.2. Decontamination efficiency of continuous and pulsed ultrasounds provided under uncontrolled temperature regime

In order to study the decontamination potential of combined ultrasound processing with *in situ* generated heat, wastewater obtained by washing fresh-cut lamb's lettuce was subjected to ultrasound treatments under uncontrolled temperature regime. To this purpose, sample temperature was left to rise during the ultrasound process due to heat dissipation. Trials without temperature control were performed in pulsed mode or continuous mode. In the former case, samples were subjected to pulsing at 0.5/0.5 seconds on/off. This modality has been already used to allow to contain the temperature rise during ultrasound process (Madge & Jensen, 2002; Bermúdez-Aguirre & Barbosa-Cánovas, 2012). Figure 4.2 shows the time-temperature profiles of wash water during continuous or pulsed ultrasound without temperature control. As expected, temperature increased during treatments, reaching approximately 90 °C after 15 min of continuous ultrasound, whereas temperature values not exceeding 65 °C were recorded for the pulsed modality. In fact, pulsed ultrasound decreased the temperature rise compared with continuous ultrasound, because the "off" interval period allowed heat to be dissipated (Madge & Jensen, 2002). The energy densities transferred into the wastewater sample during the pulsed and continuous ultrasound processes were of 205 and 572 W/L, respectively. Accordingly, the acoustic energy density values ranged between 60 and 244 kJ/L, and 32 and 687 kJ/L for the pulsed and continuous ultrasound modalities, respectively.

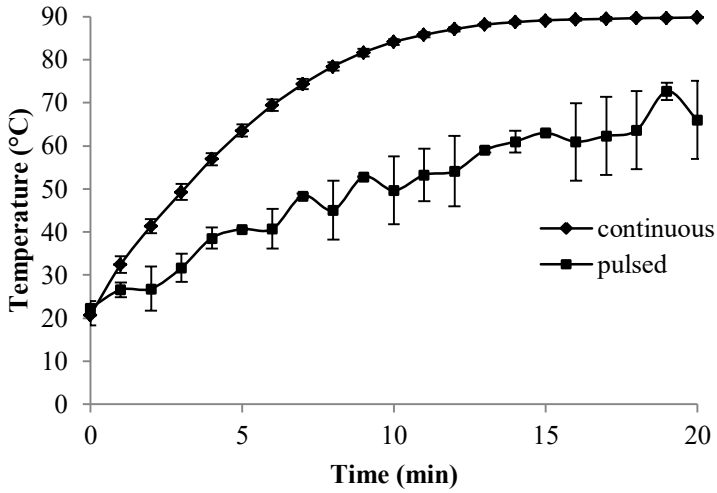


Figure 4.2. Time-temperature profiles of wastewater from fresh-cut lamb's lettuce washing during pulsed or continuous ultrasound provided under uncontrolled temperature regime.

Figure 4.3 shows the effect of pulsed and continuous ultrasound provided under uncontrolled temperature regime on the total microbial count of the wastewater obtained by fresh-cut lettuce washing. The effect of heat only, i.e. generated by providing the water sample the same time-temperature combinations received during the continuous ultrasound without temperature control, on the native microflora is also shown. The Log reductions of the total microbial count of wastewater increased linearly with exposure time ($p<0.05$). In particular, the rate constants computed from the slopes of the linear regression of the logarithm of total microbial count *vs* exposure time were 0.109, 0.147 and 0.142 min^{-1} ($0.711<R^2<0.874$) for the pulsed ultrasound, continuous ultrasound and heating, respectively. It can be observed that the rate constants of the pulsed and continuous ultrasound increased with increasing levels of power density (205 and 572 W/L, respectively), in agreement with previous findings (Gao et al., 2014; Patil et al., 2009). Thus, the lowest Log reductions were attained during pulsed ultrasound. In fact, 20 min of this treatment resulted in 2.4 Log reductions of the total viable count. According to the classification suggested by Madge and Jensen (2002), this value accounts for a good disinfection efficiency of the pulsed ultrasound. It is noteworthy that the same Log reduction was achieved by applying continuous ultrasound with temperature control (Figure 4.1). It could be argued that the additional thermal effect produced during the pulsed treatment is likely to compensate the lower cavitation effect generated during the continuous ultrasound process at controlled temperature regime. Microorganisms responded similarly to the continuous ultrasound and heating only (Figure 4.3). Twenty min application of both treatments allowed a 3.2 Log reduction of the native microflora to be achieved, thus indicating that the *in situ* generated heat contributed to microbial inactivation, in agreement with previous findings (Gómez-

López et al., 2015; Madge & Jensen, 2002; Salleh-Mack & Roberts, 2007). Overall, data reported here suggest that cavitation may be not the only mechanism of microbial decontamination. Besides physical (i.e. extreme pressure variations and micro-streaming) and chemical (i.e. formation of free radicals and H_2O_2) mechanisms, temperature rise, occurring during ultrasound, plays an important role towards microbial inactivation.

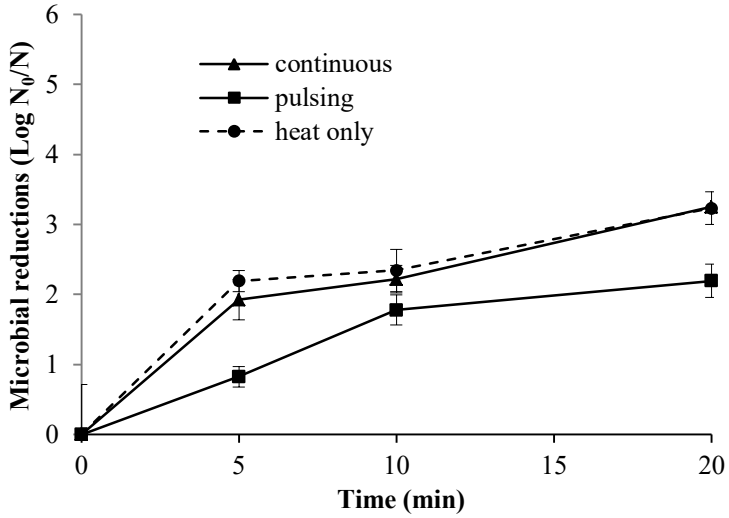


Figure 4.3. Log reductions of total microbial count in wastewater fresh-cut lamb's lettuce washing subjected to pulsed or continuous ultrasound under uncontrolled temperature regime, or heating. The latter provided the water sample the same time-temperature combinations received during the continuous ultrasound.

Figure 4.4 shows the Log reductions of the total microbial count in the wastewater derived from washing fresh-cut lettuce as a function of the specific energy generated upon the pulsed and continuous ultrasound processes without temperature control as well as heating only. As the specific energy brings together transferred power, time of exposure and treated volume (Hulsmans et al., 2010), it was used as a reference parameter to make possible the comparison. It can be observed that the plots describing the effect of pulsed and continuous ultrasound on the total viable count were almost overlapping, indicating that ultrasound modality (and thus power transferred into the fluid) had barely an effect on the microbial decontamination level, provided that the same energy (and temperature) was achieved. These two plots were in turn nearly on top of that describing the effect of the heating only on the naturally present microflora. Our results are partially in disagreement with those reported by Madge and Jensen (2002) for fecal coliforms in domestic wastewater. In fact, according to these authors, the disinfection efficiency of pulsed and continuous ultrasound was similar up to 60 kJ/L, while the pulsed ultrasound resulted less effective than the continuous treatment at increasing doses. The results of the present study clearly show that the specific energy transferred to the system during ultrasound without temperature control affected the

microbial reduction, regardless the ultrasonication modality (pulsed or continuous), and confirmed that the *in situ* generated heat contributed to decontamination.

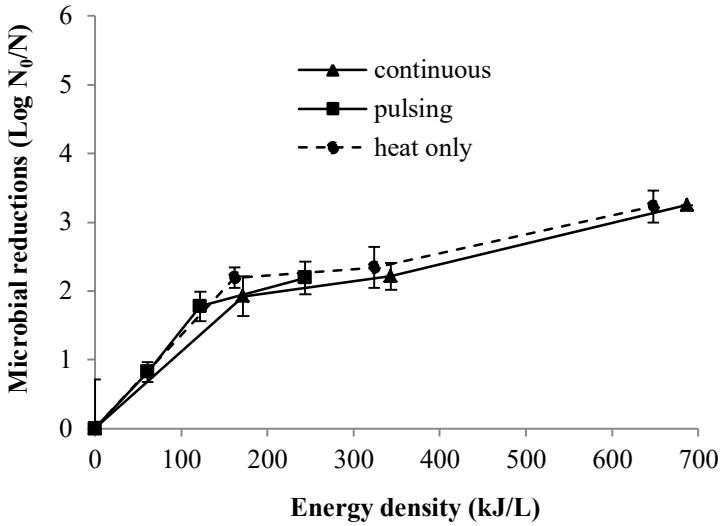


Figure 4.4. Log reductions of total microbial count in wastewater from fresh-cut lamb's lettuce washing as a function of the energy density generated upon pulsed and continuous ultrasound without temperature control as well as upon heating provided according to the same time-temperature combinations received during the continuous ultrasound.

Figure 4.5 shows the decontamination efficiency of continuous ultrasound under uncontrolled temperature regime on wastewater inoculated with *L. monocytogenes, E. coli* and *S. enterica* suspensions having initial concentration of approximately 10^6 CFU/mL. Reductions of 1.0, 1.2 and 5 Log units of *L. monocytogenes, E. coli* and *S. enterica* were attained after 3 min of continuous ultrasound, respectively. Complete inactivation of *L. monocytogenes, E. coli* was achieved at 5 min of ultrasound exposure. By subjecting wastewater inoculated with *E. coli* and *S. enterica* to heating only, by providing the same time-temperature combinations received during the continuous ultrasound, 5 Log reductions were also achieved within 5 min and 3 min, respectively. On the contrary, only 1.7 Log reductions *L. monocytogenes* were attained after 5 min heating, while complete inactivation was achieved following 10 min treatment (Figure 4.5). It must be pointed out that in our experimental conditions, temperature never exceeded 50 °C within 3 min of ultrasonication. At this sub-lethal temperature, *L. monocytogenes* cells were subjected to the ultrasound effect only. On the contrary, as at 5 min of treatment the temperature rose to 65 °C, a contribution to *L. monocytogenes* reduction of the heat generated during the ultrasound process above this exposure time can be inferred, in agreement with previous studies (Bauman, Martin, & Feng, 2005; Gómez-López et al., 2014; Pagan et al., 1999; Salleh-Mack & Roberts, 2007). Results indicate that the same decontamination efficiency against *E. coli* and *S. enterica* was achieved by providing either ultrasound or heating processes. Only in the case of *L.*

monocytogenes different contributions to microbial reduction were found for ultrasound without temperature control and heating only.

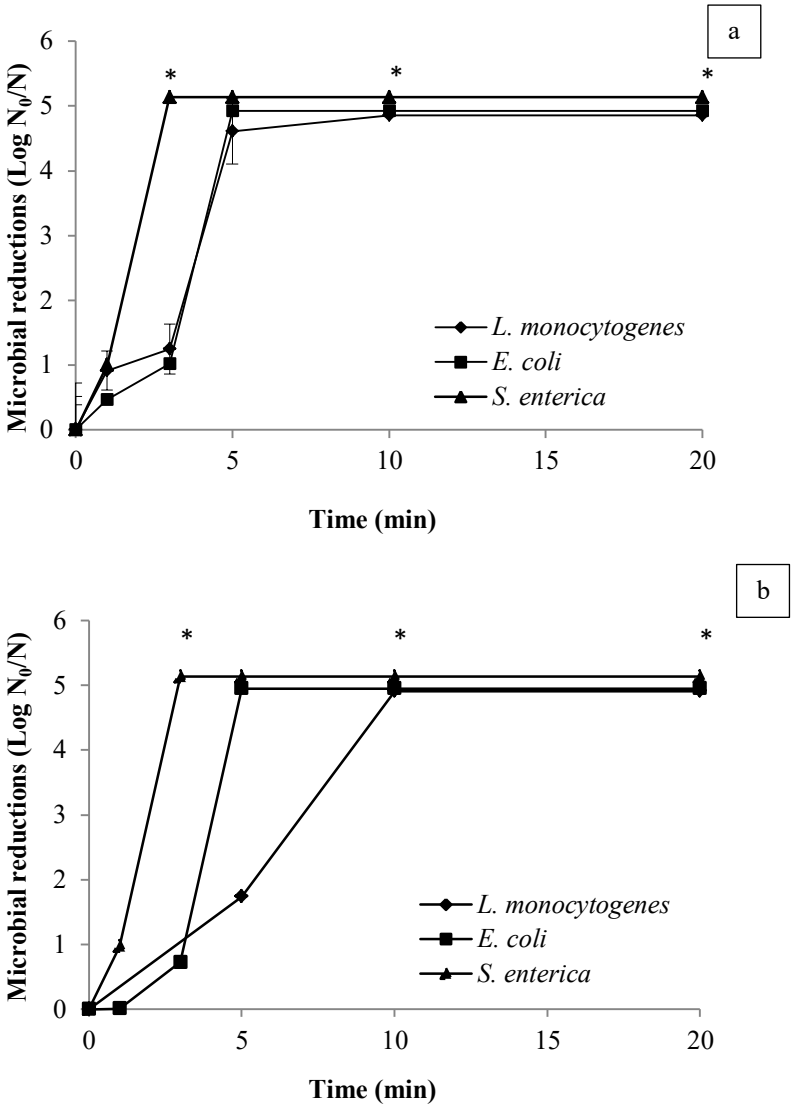


Figure 4.5. Log reductions of *L. monocytogenes, E. coli* and *S. enterica* inoculated in wastewater from fresh-cut lamb's lettuce washing as a function of time for (a) continuous ultrasound under uncontrolled temperature regime, (b) heat treatment only.
*: counts below the detection limit of 1 Log CFU/mL.

To actually differentiate cavitation and heat contributions to bacteria inactivation, *L. monocytogenes, E. coli* and *S. enterica* logarithmic cell numbers in wastewater samples were compared in terms of energy density provided during either the continuous ultrasound treatments with or without temperature control or heating. Table 4.1 shows

the rate constants computed from the slopes of the linear regression ($p<0.05$) of the logarithm of bacterial count *vs* energy values (kJ/L), and the correspondent determination coefficients.

Table 4.1. Rate constants computed from the slopes of the linear regression of the logarithmic cell number of *L. monocytogenes, E. coli* and *S. enterica* in wastewater from fresh-cut lamb's lettuce washing subjected to continuous ultrasound processing (US) with or without temperature control or heating vs energy density values (kJ/L), and correspondent determination coefficients.

	US with temperature control		**US without temperature control**		**Heat only**	
	k (L/kJ)	**R^2**	**k (L/kJ)**	**R^2**	**k (L/kJ)**	**R^2**
L. monocytogenes	0.057	0.830	0.0263	0.858	0.0152	0.967
E. coli	0.0125	0.979	0.0278	0.892	0.0298	0.843
S. enterica	0.0144	0.889	0.0449	0.965	0.0477	0.963

The estimated inactivation rate constant for *L. monocytogenes* in wastewater subjected to ultrasound without temperature control was greater than the inactivation rate constants obtained by either heating only or ultrasound under controlled temperature regime. According to Madge and Jensen (2002), these rate constants were used to determine the acoustic and thermal contributions to disinfection. In particular, the former was calculated as the percentage ratio of the rate constants of ultrasonication with and without temperature control; the thermal contribution was computed as the percentage ratio of the rate constants of thermal treatment and ultrasound process without temperature control. The acoustic and thermal contributions to *L. monocytogenes* inactivation were estimated to account for about 22 and 58%, respectively. The remaining 20% of unaccounted contribution can be attributed to synergistic effects. These results are in agreement with data reported by Madge and Jensen (2002) for fecal coliform bacteria in domestic wastewater subjected to ultrasound treatment at 700 W/L with or without temperature control and heating only. Data of Table 4.1 also show that the estimated values of inactivation rate constants for *E. coli* and *S. enterica* subjected to continuous ultrasound without temperature control were almost the same of those accounting for the heat treatment only. In other words, a small temperature rise (i.e. from 30 °C to 50 °C for *S. enterica*; from 30 °C to 63 °C for *E. coli*) allowed the disinfection efficiency to be greatly increased. Therefore, in our experimental conditions, the effectiveness of continuous ultrasound carried out without temperature control compared with that provided under controlled temperature regime against *E. coli* and *S. enterica* was mainly due to the thermal contribution, while the acoustic mechanism was negligible. Differences in acoustic and heat contributions observed among *L. monocytogenes, E. coli* and *S. enterica* can be brought back to their different sensitivity to heat and ultrasounds, *L. monocytogenes* being the most resistant (Pagan et al., 1999).

To find whether these treatments had reversible or irreversible effects, resuscitation trials were carried out on *L. monocytogenes, E. coli* and *S. enterica* inoculated wastewater already subjected to continuous ultrasound without temperature control or heat treatment. Results showed that *E. coli* and *S. enterica* were irreversibly inactivated by 5 min of both treatments, whereas *L. monocytogenes* cells, although stressed, were able to re-grow, indicating their ability to repair the cellular damage. However, no resuscitation was observed for *L. monocytogenes* cells subjected to longer treatments.

4.4. Conclusions

The results acquired in this study highlighted the effectiveness of pulsed and continuous ultrasounds in decontaminating wastewater derived from fresh-cut production. When ultrasound was provided with temperature control, different capabilities were found among the microorganisms considered (i.e. native microflora as well as inoculated *L. monocytogenes, E. coli* and *S. enterica)* to withstand physical and chemical effects of cavitation, *L. monocytogenes* and *S. enterica* being the most and the least resistant, respectively. When ultrasound was applied without temperature control, a 5 Log reduction of the pathogenic bacteria was achieved within 5 min. Such a rapid decontamination was attributed to the contribution of *in situ* generated heat during ultrasound treatment. The thermal contribution accounted for 58% for *L. monocytogenes*, while it represented the prevalent mechanism for *E. coli* and *S. enterica*, that are more heat sensitive bacteria. In light of this, instead of increasing ultrasound power input and dissipate the heat produced during the treatment, it seems more feasible to apply lower acoustic power densities and exploit the *in situ* generated thermal effect to decontaminate wastewater obtained by fresh-cut vegetable washing from heat resistant microorganisms.

In the attempt to optimize the wastewater management in the fresh-cut sector, application of ultrasounds in combination with *in situ* generated heat to wastewater decontamination could represent a promising tool for water recycling inside a fresh-cut production. Therefore, this technology could be an alternative strategy to the addition of disinfectants (mainly chlorine compounds) that are currently used to avoid microbial proliferation and vegetable cross contamination by spoilage and pathogenic microorganisms. Moreover, besides safety requirements, this technology would also meet cost-effectiveness criteria and existing standards.

Part II

Effect of unconventional technologies on functionality of fruit and vegetable derivatives

Chapter 5

Application of HPH treatment for delivering silybin in oil-in-water nanoemulsions

HPH treatment induced changes of some physical and chemical properties of food molecules (Donsì et al., 2013; Fathi, Martin, & McClments, 2014; Floury et al., 2000). In particular, HPH treatments have been proposed as alternative techniques to produce nanoemulsions (10–100 nm radius) since they can impart a sufficiently high energy input to reduce the droplet dimensions at nano-level of oil-in-water mixtures (McClements, 2005). According to Rao and McClements (2012), nanoemulsions can be effectively used to incorporate poorly water-soluble nutraceuticals in functional foods. However, the choice of the lipid medium appears particularly critical, since the chemical and physical characteristics of the lipid carrier greatly affect the solubility of the compound to be delivered.

Silymarin is a mixture of flavolignans (i.e. silybin, silydianin and silychristin) extracted from *Silybum marianum*. Among the flavolignans, silybin or silybinin is the most abundant biologically active compound of silymarin. The use of silymarin to treat liver diseases, such as cirrhosis, hepatitis, alcoholic liver disease and toxin exposure has been well documented (Flora et al., 1998; Franceschini, Demartini, & Esposti, 2002). Several studies investigated how to develop pharmaceutical preparations in order to increase silybin bioavailability, on the other hand, less attention has received its incorporation into food.

5.1 Aim of the study

The aim of this research was to study the potential for nanoemulsion delivery systems to carry silybin from silymarin extract. To this purpose, HPH treatment was applied and different carrier oils (sunflower oil, extra virgin olive oil and castor oil) were used to prepare silymarin loaded nanoemulsions. The physical and chemical stability of nanoemulsions was studied during storage at 20 °C. Also, the effect of oil type on the silybin in *vitro* bioaccessibility was evaluated.

5.2. Materials and methods

5.2.1. Silybin solubility

Aliquots of 3 mL of sunflower oil, castor oil, extra virgin olive oil, Tween 80 (Sigma Aldrich, St. Luois, MO, USA) or deionized water were introduced in 5.0 mL capacity vials and excess amount of silymarin extract, containing 210 mg/g of silybin, 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH·), was added. Similarly, 1.5 g of each oil and 1.5 g of Tween 80 were added with excess amount of silymarin extract in 5.0 mL vials. Samples were kept at a constant temperature (25 ± 1.0 °C) under shaking for 72 h to reach equilibrium (Parveen et al., 2011). The samples were centrifuged at 13100 g for 10 min (MiniSpin, Eppendorf, Hamburg, Germany) and the solubilised silybin in the supernatant was then recovered and quantified by HPLC analysis (Paragraph 5.2.3.2).

5.2.2. Nanoemulsion preparation

The oil phase was prepared by mixing silymarin powder (2.5 mg/g) and the surfactant Tween 80 (10 mg/g) in sunflower oil, extra virgin olive oil or castor oil. Preliminary trials evidenced that this surfactant concentration allowed a stable emulsion to be obtained. The systems were stirred in the dark until the silymarin was completely dissolved. No recrystallization events were observed before emulsion preparation. The aqueous phase consisted of deionised water added with 0.1 mg/g of sodium azide (Sigma Aldrich, St. Luois, MO, USA), to avoid microbial spoilage during the storage experiments. The stock emulsions were prepared by mixing 200 g/L oil phase with the aqueous phase with a high speed blender for 1 min at 9000 rpm (Polytron, PT 3000, Cinematica, Littau, Swiss). Aliquots of 250 mL of the stock emulsions were homogenised at at 150 MPa by using the same equipment reported in paragraph 2.2.3.1 (pag. 22). Aliquots of 18 mL of the nanoemulsions were inserted into 20 mL colourless glass vials, sealed with butyl septa and metallic caps and stored at 20 °C in a thermostatic cell for approximatively 50 days.

5.2.3. Analytical determinations

5.2.3.1. Particle size

The mean diameter of emulsion droplets was measured by using the dynamic light scattering instrument Particle Sizer NICOMP™ 380 ZLS (PSS NICOMP Particle Sizing System, Santa Barbara, California, USA). Samples were diluted 1:1000 (v/v) with deionised water prior to the analysis to avoid multiple scattering effects. The angle of observation was 90°. Solution refractive index and viscosity were set at 1.333 and 1.0 cP, respectively, corresponding to the values of pure water at 20 °C. Particle mean diameter corresponding to volume distribution was calculated by NICOMP Distribution Analysis.

5.2.3.2. Silybin concentration

Silybin extraction was performed by introducing 1 g supernatant or nanoemulsion into 10 mL Pyrex tubes, added with 5 mL water:methanol mixture (1:4 v/v), and manually shaken for 2 min. The tubes were then treated for 15 min in an ultrasonic bath (25 °C) and finally centrifuged at 1000 g for 10 min (Labofuge I, Heraeus Christ GmbH, Osterode am Harz, Germany). Samples were then stored overnight at −20 °C, to improve the phase separation. The upper water-methanol phase was filtered on 0.20 µm pore size nylon membranes (Albet-Hahnemühle, Barcelona, Spain), and analysed for silybin concentration by reverse-phase HPLC according to the slightly modified method of Kvasnička et al. (2003). Analyses were performed by an LC-2010 AHT liquid chromatographic system (Shimadzu, Kyoto, Japan) equipped with an integrated UV–visible detector. A 4 µm packed 150 × 4.6 mm C18 column (Synergi Polar, Phenomenex, Torrance, CA), thermostated at 35 °C, was used. The elution was in gradient mode using a mixture of 5 mL/L aqueous phosphoric acid (solvent A) (Carlo Erba Reagents, Milan, Italy) and methanol (solvent B) (Carlo Erba Reagents, Milan, Italy) as mobile phase at a flow rate of 1 mL/min. Gradient was set as follows: solvent B was held at 36% for the first 5 min, increased to 45% in 1 min and held at this level for

25 min; then 100% solvent B was reached in 2 min and held for 5 min, before to be lowered in 2 min to the initial level (36%). The sample injection volume and the detection wavelength were 10 μL and 288 nm, respectively. Quantitative analysis was carried out by comparing the silybin peak area with the results of a calibration line, obtained by injecting silybin standard solutions (methanol:water 4:1 v/v) (Extrasynthese, Genay, France). Calibration curve was linear (R^2=0.999) in the 0.5–18.0 mg/L concentration interval.

5.2.3.3. Chain breaking activity

The chain-breaking activity was measured following the methodology of Brand-Williams, Cuvelier, and Berset (1995). The bleaching rate of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH·) was monitored at 515 nm. A volume of 1.85 mL of 6.1×10^{-5} mol/L DPPH· methanol solution was used. The reaction was started by the addition of 150 μL of sample, previously solubilised with methanol. The DPPH· bleaching was followed at 515 nm (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for at least 10 min. In all cases the DPPH· bleaching rate was proportional to the sample concentration added to the medium. The reaction rate of DPPH· bleaching was computed according to the following equation (Manzocco, Anese, & Nicoli, 1998):

$$\frac{1}{A^3} - \frac{1}{A_0^3} = 3kt \qquad \text{(eq. 5.1)}$$

where k is the DPPH· bleaching rate, A_0 is the initial absorbance value, and A is the absorbance at increasing time, t. The chain-breaking activity was expressed as the slope (k) obtained from eq. 5.1 per milligram of dry matter (A^{-3}/min·g dm), assuming that all of the sample dry matter possessed antioxidant capacity.

5.2.3.4. Oxygen concentration

Oxygen concentration was measured by an OxySense® fluorimeter (OxySense Inc., Dallas, TX, USA). Aliquots of 18 mL of the nanoemulsions were introduced into 20 mL colourless glass vials. Preliminarily, an oxygen sensitive sensor (O2xyDot®, OxySense Inc., Dallas, TX, USA) was pasted on approximately 1 cm from the bottom edge of the internal surface of the vials, by using an oxygen permeable glue (OxySense Inc.). When the sensor is illuminated by a pulsed blue light, a red fluorescent light is emitted, that is measured by the fluorimeter. The decrease of the O2xyDot® fluorescence lifetime, due to dynamic oxygen quenching, is proportional to the oxygen concentration in the sample. Sample temperature was measured simultaneously, by a sensor located in the reader pen of the fluorimeter (Li et al., 2008). Results were expressed as percentage of residual oxygen.

5.2.3.5. Lipid hydroperoxide concentration

Lipid hydroperoxide concentration was determined following the methods of Shantha and Decker (1994) and Katsuda et al. (2008). In particular, 3 mL nanoemulsion were mixed three times with 15 mL isooctane:2-propanol (3:1) solution and vortexed for 10 s.

After centrifugation for 2 min at 2000 g, the clear upper layer was collected (0.20 mL) and mixed with 2.8 mL methanol:1-butanol (2:1) solution, 15 µL of 3.94 mol/L ammonium thiocyanate solution and 15 µL of 0.0072 mol/L ferrous ion solution (prepared through the mixture of 0.132 mol/L $BaCl_2$ and 0.144 mol/L $FeSO_4$). After 20 min incubation at room temperature, absorbance was measured at 510 nm with a spectrophotometer (Shimadzu, UV-2501PC, Japan). Hydroperoxide concentration was determined using cumene hydroperoxide (Sigma Aldrich, St. Luois, MO, USA) standard curve.

5.2.3.6. Silybin in vitro *bioaccessibility*

In vitro intestinal digestion. A dynamic *in vitro* digestion model was used to study the influence of emulsion composition on silybin bioaccessibility. The methods of Zangerberg et al. (2001a and 2001b) and Mun et al. (2006) were followed with some modifications. 0.75 mL nanoemulsion was mixed with phosphate buffer (pH 7) and heated at 37 °C for 10 min in a water bath. Then, the pH of the sample was adjusted to 7.00 with 2 mol/L NaOH and 4 mL bile extract (46.9 g/L in phosphate buffer at pH 7) (Sigma Aldrich, St. Luois, MO, USA) and 1 mL calcium chloride (110 g/L in deionized water) (Sigma Aldrich, St. Luois, MO, USA) were added. The pH was adjusted to 7.00 if necessary. Finally, 2.5 mL of freshly prepared lipase suspension (24 g/L in phosphate buffer at pH 7) (Sigma Aldrich, St. Luois, MO, USA) was added to the mixture. The pH of the mixture was monitored and maintained at 7.00 by adding 0.1 mol/L NaOH. The volume of NaOH added to the sample was recorded and used to calculate the concentration of free fatty acids (FFA) generated during lipolysis. The extent of lipolysis was determined as follows:

$$FFA\ released\ (\%) = \frac{NaOH\ amount\ consumed}{theoretical\ NaOH\ amount\ for\ complete\ lipolysis} . 100 \qquad \text{(eq. 5.2)}$$

To calculate the volume of NaOH required for complete lipolysis, it was assumed that 1 molecule of sunflower oil, extra virgin olive oil or castor oil consumed 2 molecules of NaOH (Yu & Huang, 2012), each oil molecule being hydrolysed by pancreatic lipase into two free fatty acids and one monoacylglycerol molecule.

In vitro *bioaccessibility determination*. The *in vitro* bioaccessibility of silybin was evaluated after the in *vitro* digestion was completed. The digest was immediately centrifuged (XL-70 Ultracentrifuge, Beckman, Palo Alto, CA, USA) at 165000 g at 4 °C for 70 min. After centrifugation, the sample was separated into an opaque sediment phase (pellet) and a clear phase containing the mixed micelles (supernatant). Silybin was extracted from the micelles by liquid–liquid extraction. Briefly, 20 mL digest was introduced in 50 mL Pyrex tubes and mixed with 200 µL of 218.8 g/L hydrochloric acid and 100 µL of naringenin-7-O-glucoside methanol solution (0.73 g/L) (Extrasynthese, Genay, France). 10 mL ethyl acetate was then added and the tubes were subjected to manual shaking (5 min) followed by immersion in ultrasonic bath (1 h, 40 °C). The sample was then centrifuged (1000 g, 10 min) and the organic phase separated. Manual

extraction and ultrasound treatment were repeated twice and the ethyl acetate extracts concentrated to a final volume of approximately 1 mL, in a vacuum centrifuge (Univapo 100 H, UniEquip GmbH, Freital, Dresden, Germany). Silybin quantification in the concentrated extracts was carried out by HPLC as reported above (Paragraph 5.2.3.2). The bioaccessibility (%) was defined as the percentage ratio between silybin concentration in the mixed micelles and the silybin concentration in the digest. In addition, to allow a better traceability of the repartition of the active compound between the pellet and micelles, the pellet was also analysed for silybin content. To this purpose, the sediment was re-suspended in 5 mL methanol and 100 µL 218.8 g/L hydrochloric acid. Extraction was performed by manual shaking (5 min) followed by immersion in ultrasonic bath (40 °C for 1 h). Samples were finally filtered on 0.20 µm pore size nylon membranes (Albet-Hahnemühle, Barcelona, Spain), and silybin content was determined by HPLC as reported above (Paragraph 5.2.3.2). Mass balance of silybin in pellet and micelles evidenced the complete recovery of the bioactive compound.

5.2.4. Statistical analysis

The results are the average of at least two measurements carried out on two replicated experiments ($n \geq 4$). Data are reported as mean value ± standard deviation. Statistical analysis was performed using Statistica for Windows ver. 5.1 (Statsoft Inc. Tulsa, USA, 1997) Bartlett's test was used to check the homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p<0.05$).

5.3. Results and discussion

5.3.1. Silybin solubility

The solubility of silybin in water, selected oils and Tween 80 is shown in Table 5.1. In accordance with the literature, the solubility of silybin in water was negligible (Gazak et al., 2004 and Yang et al., 2013). Silybin presented the highest solubility in castor oil followed by extra virgin olive oil and sunflower oil. As known, among the selected oils, castor oil is the most polar oil due to its high content of ricinoleic acid. This result is consistent with the data reported by Yang et al. (2013) and seems to indicate that a certain degree of polarity of the oil phase might favour silybin solubility. Data on the surfactant Tween 80 support this hypothesis showing silybin solubility one order of magnitude higher than in the oils. As reported in the literature, Tween 80 is actually used as surfactant in different nanoemulsions for drug delivery (Parveen et al., 2011; Yang et al., 2013). Furthermore, silybin solubility was determined in mixes of sunflower oil, extra virgin olive oil or castor oil, and Tween 80 in 1:1 (v/v) ratio. Results showed that in all mixtures the silybin solubility had a value comparable to that found in the Tween 80 alone (Table 5.1).

Table 5.1. Silybin solubility obtained in water, sunflower oil, extra virgin olive oil, castor oil, Tween 80, Tween 80-sunflower oil (1:1 v/v), Tween 80-extra virgin olive oil (1:1 v/v) and Tween 80-castor oil (1:1 v/v). Results were obtained by adding an excess amount of silymarin extract in each system and after 72 h of equilibration at 25 °C.

Medium	Silybin (mg/g)
Water	nd
Sunflower oil	0.028 ± 0.006^{b}
Extra virgin olive oil	0.009 ± 0.002^{a}
Castor oil	0.668 ± 0.072^{c}
Bulk Tween 80	2.061 ± 0.110^{d}
Tween 80-sunflower oil	1.987 ± 0.134^{a}
Tween 80-extra virgin olive oil	2.221 ± 0.205^{a}
Tween 80-castor oil	1.555 ± 0.310^{a}

^{a,b,c,d}*: means with different letters are significantly different ($p<0.05$)*
nd: not detectable

To compare the performances of different nanoemulsions to carry silybin, 2.50 mg/g silymarin extract, corresponding to 0.525 mg/g silybin, was added to sunflower oil, extra virgin olive oil or castor oil containing 10 mg/g Tween 80 before emulsion preparation. At this level, silybin was completely solubilized in the mixture (Table 5.1).

5.3.2. Physical properties of silymarin enriched nanoemulsions

Table 5.2 shows the mean particle diameter of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin extract. Control samples prepared without silymarin showed results not significantly different from the bioactive enriched counterparts (data not shown). Although the samples had relatively small mean particle diameters, appreciable differences in the values among the three types of nanoemulsions can be observed, the one prepared with castor oil showing higher values. As emulsion properties are greatly affected by the nature of the oil used (McClements, 2005), these results can be attributed to differences in chemical properties among the oils considered. In particular, the higher particle size of nanoemulsion with castor oil can be due to higher viscosity and polarity of castor oil in comparison with extra virgin olive oil and sunflower oil (Jaworska et al., 2014; Qian and McClements, 2011). Neither significant increase in nanoemulsions particle size nor visible sediments at the bottom of the test vials were found during storage at 20 °C for up to 50 days. These results suggest that silymarin components did not undergo separation and re-crystallisation phenomena during storage.

Table 5.2. Mean particle diameter of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions containing Tween 80 as surfactant (20 mg/g nanoemulsion), enriched with silymarin extract.

Storage time (days)	Mean particle diameter (nm)		
	Sunflower oil	**Extra virgin olive oil**	**Castor oil**
0	208±39[a]	241±46[a]	307±52[b]
15	208±37[a]	229±39 [a]	309±53[b]
36	208±38[a]	235±48[a]	329±6[b]
50	232±49[a]	235±47[a]	323±62[b]

[a,b]*: means with different letters in the same row are significantly different ($p<0.05$)*

5.3.3. Silybin in vitro *bioaccessibility of silymarin enriched nanoemulsions*

The effect of carrier oil on the silybin *in vitro* bioaccessibility in nanoemulsions was studied. As well known, triacylglycerols have to be decomposed into free fatty acids and monoglycerides to allow the bioactive molecule silybin to be released and subsequently incorporated into the mixed micelles, i.e. made of bile salts and lipolytic products, to be transferred to the epithelium cells (Hofmann & Borgstrom, 1964). To monitor the rate and extent of lipid digestion, the formation of free fatty acids from the nanoemulsions during simulated small intestine digestion was measured (Figure 5.1). The free fatty acids release steadily increased in the first minutes of the digestion, suggesting that the lipase promptly attached to the oil droplets surface due to an efficient displacement of the surfactant layer by the bile salts (Qian, Dereck, Xiao, & McClements, 2012). In our experimental conditions, the free fatty acids release during the *in vitro* intestinal digestion was not affected by the carrier oil type, which is consistent with previous results (Hur et al., 2011). The oils used were actually all composed of long chain fatty acids, the chain length being an influencing factor of the extent and rate of in vitro lipolysis. Moreover, our results clearly show that in all cases the free fatty acids release was almost complete (around 80–90% digestion).

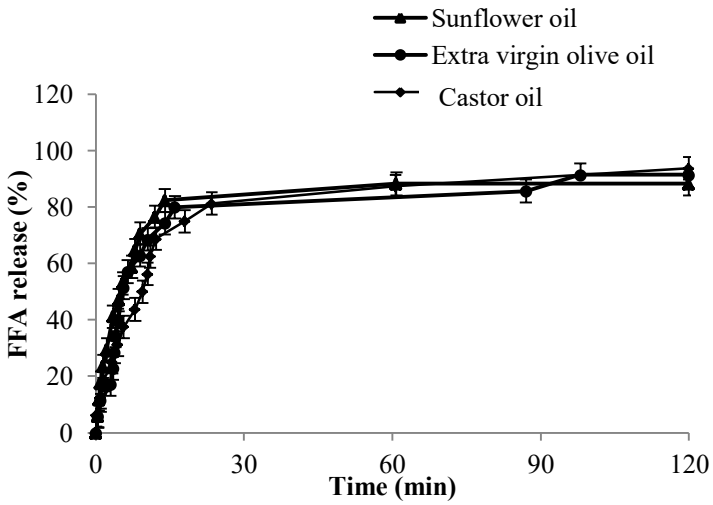


Figure 5.1. Free fatty acids (FFA) release (%) from sunflower oil, extra virgin olive oil and castor oil based nanoemulsions containing Tween 80 as surfactant (20 mg/g in nanoemulsion), enriched with silymarin extract. Bars represent the standard deviation of two replicated experiments made in duplicate.

The silybin concentration in the mixed micelles (i.e. silybin bioaccessibility) and precipitated pellets obtained from the digesta were then measured (Table 5.3). Results showed that the carrier oil only slightly influenced the silybin *in vitro* bioaccessibility. In fact, independently of oil type, silybin concentration in the micelles was approximately 25–30%. These results indicate a beneficial effect of the use of nanoemulsions for the bioaccessibility of silybin.

Table 5.3. Percentage of silybin in mixed micelles (i.e. *in vitro* bioaccessibility) and pellet after *in vitro* digestion of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions containing Tween 80 as surfactant (20 mg/g nanoemulsion) enriched with silymarin extract.

Carrier oil	**Silybin (%)**	
	Micelles	**Pellet**
Sunflower oil	25.3±2.1^{b}	78.1±6.5^{a}
Extra virgin olive oil	29.1±0.7^{a}	71.1±4.5^{ab}
Castor oil	29.6±1.6^{a}	68.0±0.6^{b}

$^{a-b}$: means with different letters are significantly different ($p < 0.05$)

5.3.4. Chemical properties of silymarin enriched nanoemulsions

Despite a huge number of papers deal with the mechanisms of biological activity of silybin and other silymarin components, to our knowledge, the effect of flavonoids from silymarin on food lipid oxidation has not been well investigated. Figure 5.2 shows the changes in oxygen concentration of sunflower oil, extra virgin olive oil and castor oil containing nanoemulsions enriched with silymarin as a function of storage time at 20 °C.

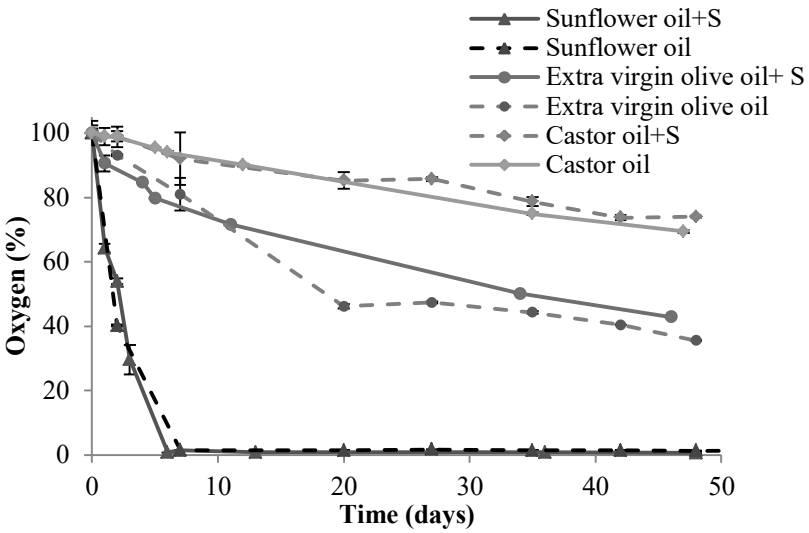


Figure 5.2. Percentage of residual oxygen in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions containing Tween 80 as surfactant (20 mg/g in nanoemulsion), enriched with silymarin extract (continuous line) or not (dotted line) as a function of storage time at 20 °C. Bars represent the standard deviation of two replicated experiments made in duplicate.

Nanoemulsions not containing silymarin extract were used as controls. Oxygen concentration decreased faster in nanoemulsions containing sunflower oil, followed by nanoemulsions with extra virgin olive oil and castor oil. Such a reactivity rank is consistent with the unsaturation degree of the incorporated oils, sunflower oil having a higher value than extra virgin olive oil, which in turn has a greater number of carbon–carbon double bonds than castor oil. No significant differences in oxygen concentration were found between nanoemulsions with and without silymarin extract. This result suggests that silymarin components did not act as oxygen scavengers, in agreement with the pionering data reported by Dehmlow et al. (1996), who found a negligible capacity of silybin to react with species. Moreover, the hydroperoxide concentration of nanoemulsions with or without silymarin extract changed during storage with different kinetics depending on the nature of the carrier oil, the sunflower oil and castor oil containing nanoemulsions being the most and the least susceptible to oxidation, respectively (Figure 5.3). However, also in this case, the evolution of hydroperoxides in the nanoemulsions enriched with silymarin did not significantly differ from those of the

respective nanoemulsions without the bioactive molecule. These results further indicate that silymarin incorporation into nanoemulsions did not influence the pathway of the oxidative reactions occuring in oils. However, they contrast with data on the liver protective effect of silymarin components, that is generally attributed to the antioxidant activity of the bioactive molecule (Shaker, Mahmoud, & Mnaa, 2010).

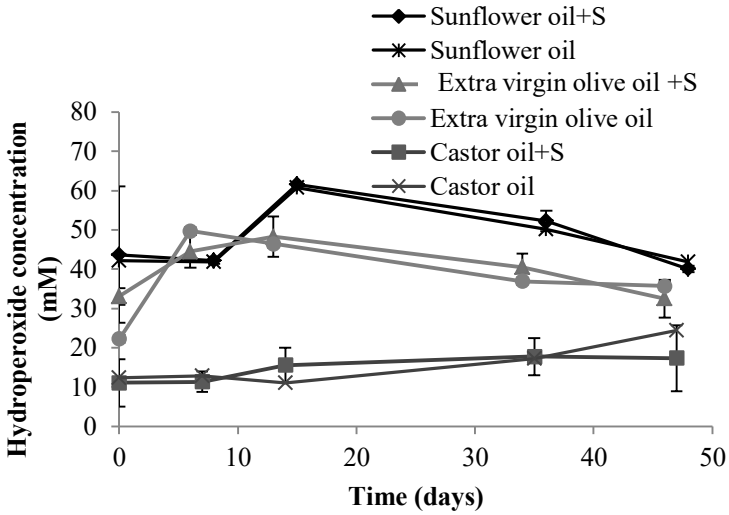


Figure 5.3. Changes in hydroperoxide concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions containing Tween 80 as surfactant (20 mg/g in nanoemulsion), enriched with silymarin extract (continuous line) or not (dotted line) as a function of storage time at 20 °C. Bars represent the standard deviation of two replicated experiments made in duplicate.

To have insights on the chain breaking activity of silymarin extract used in this study, the DPPH· assay was performed. Only a weak ability of silymarin to scavenge the DPPH· radical was measured (i.e. 0.14 ± 0.06 A^{-3}/min·g dm). Such a value was much lower than the chain breaking activity of α-tocopherol and Trolox, that were 0.94 ± 0.24 and 8.47 ± 0.45 A^{-3}/min·g dm, respectively. These results are in agreement with the data of the literature (Gazak et al., 2004 and Henning et al., 2014). In particular, Henning et al. (2014) reported that the *S. marianum* extracts had antioxidant properties lower than other dietary supplements, such as pomegranate, resveratol and green tea. It is noteworthy that other authors found that silymarin extract had high *in vitro* radical scavenging activity (Koksal et al., 2009).

Despite the results described above, silybin concentration in nanoemulsions greatly decreased during storage (Figure 5.4). Such a decrease was greater in sunflower oil based nanoemulsions followed by extra virgin olive oil and castor oil containing samples. In particular, approximately at 50 days storage, silybin losses were 60% and 55% in nanoemulsions containing sunflower oil and extra virgin olive oil, respectively, whereas 25% reduction of silybin content was found in the nanoemulsion with castor oil.

These discrepancies in silybin degradation kinetics might be brought back to differences in the bioactive reactivity in the media considered. It can be inferred that the lower droplets size of sunflower oil and extra virgin olive oil nanoemulsions could favour silybin degradation due to increased droplet surface area, in agreement with the literature (Gothani et al., 1999; McClements & Decker, 2000).

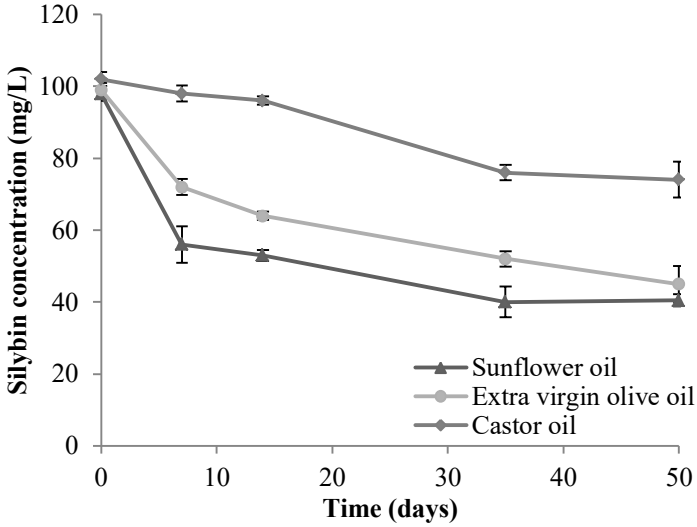


Figure 5.4. Silybin concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions containing Tween 80 as surfactant (20 mg/g in nanoemulsion), enriched with silymarin extract as a function of storage time at 20 °C. Bars represent the standard deviation of two replicated experiments made in duplicate.

Moreover, by virtue of its high affinity to Tween 80 (Table 5.1), silybin would preferably migrate at the interface of sunflower oil and extra virgin olive oil containing nanoemulsions where its degradation would be promoted (Waraho, McClements, & Decker, 2011). On the contrary, when castor oil was used as a carrier, silybin would be preferably located inside the oil droplets, being thus less prone to degradation. Possible silybin degradation by reactive species generated from oil oxidative reactions may not be ruled out. Gazak et al. (2004) actually described a number of oxidized derivatives of silybin. These compounds were obtained under strong oxidative conditions (e.g., H_2O_2 in $NaHCO_3$, iodine in glacial acetic acid, high temperature). In our experimental conditions, the greater the oil susceptibility to oxidation, and thus the formation of oxidation products, the greater the silybin degradation. It is a matter of fact that due to the scarcity of information available in the literature, further experiments are needed to understand the fate of silybin in food related environments.

5.4. Conclusion

The present study is a first attempt to develop delivery systems for incorporating silymarin into functional foods. Results showed that silybin, the main biologically active compound of silymarin, can be successfully incorporated into physically stable nanoemulsions. The oil type in the formulation slightly affected the *in vitro* bioaccessibility of the silybin, thus increasing the number of possible applications in food systems.

Although silymarin extract incorporation did not affect oil oxidative kinetics, silybin concentration in nanoemulsion decreased during storage. Such a reduction was greater in extra virgin and sunflower oil than in castor oil. This instability rank is consistent with that relevant to the susceptibility to oxidation of the carrier oils considered. Thus, the specific application of nanoemulsions in foods should take into account the effect of processing and storage conditions on silybin and oil degradation. Although additional studies should be accomplished to fully elucidate the mechanism of silybin degradation in lipid carriers, the information acquired represents an important contribution for the design and fabrication of silymarin delivering nanoemulsions. In fact, based on the results of the present study, nanoemulsions seem to represent a suitable tool for the development of functional foods and pharmaceutical products to deliver silymarin components for therapeutic purposes. It is noteworthy that further studies are required to evaluate the stability of silybin delivery nanoemulsion in foods, such as beverages, salad dressings, powder, etc.

Chapter 6

Effect of high frequency US treatments on lycopene, total phenolic concentration, antioxidant activity and α-glucosidase inhibitory activity of tomato juice

High frequency US treatments (100 kHz to 1000 kHz) are used for food quality monitoring and diagnostic purposes. However, recent studies have found that during high frequency US treatments cavitation phenomena produce hydroxyl radicals that can react with bioactive compounds, such as polyphenols and thus modify their antioxidant activity (Ashokkumar et al., 2008).
Tomatoes are well known to exert functional properties due to their high content of carotenoids and polyphenols. The latter have been recently investigated for their ability to inhibit carbohydrate hydrolysing enzyme such as α-glucosidase (Hanhineva et al., 2010). However, available data on the effect of high frequency US treatments on physical and chemical properties of tomato are still scarce.

6.1 Aim of the study

The aim of this work was to investigate the effect of high frequency (378 and 584 kHz) US treatments on the fate of bioactive compounds (lycopene and total phenolic compounds) present in tomato as well as the antioxidant properties and α-glucosidase inhibitory activity of tomato extracts. The treatment was performed for increasing length of time (up to 60 min), thus providing increasing energy densities (up to 250 MJ/m^3). The results obtained by providing the maximum energy density value were compared with those obtained for samples subjected to low frequency US treatment (24 kHz).

6.2 Materials and methods

6.2.1. Sample preparation

Commercial pasteurized tomato juice (7.5 °Brix) was sieved to separate seeds and coarse particles, and submitted to US treatment. Tomato juice not subjected to US treatment was used as a control.

6.2.2. Sonication

US treatments were conducted using two ultrasonic processors operating at either 378 kHz or 583 kHz, and 24 kHz.

6.2.3.High frequency US treatments at 378 kHz and 583 kHz

An ultrasonic processor (Meinhardt Ultraschlltechnik Leipzig, Germany) equipped with two transducers (operating at 378 kHz and 584 kHz) was used. Aliquots of 250 mL of tomato juice were introduced into a glass reaction vessel (63 mm internal diameter) with a cooling jacket (wall thickness 5 mm) connected to a cryostatic bath (Fisher Scientific, ISOTEMP Thermostatic bath). Samples were subjected to sonication for increasing

length of time up to 60 minutes. During the ultrasound treatment, the temperature never exceeded 20 °C.

6.2.4. Low frequency US treatment at 24 kHz

Samples were subjected to US treatment using the same equipment reported in paragraph 2.2.3.2 (pag. 22). Aliquots of 100 mL of tomato juice were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass vessel. The tip of the sonicator horn was placed in the centre of the tomato juice, with an immersion depth in the fluid of 50 mm. Sample were subjected to sonication up to 3 minutes. During the ultrasound treatment the temperature never exceeded 40 °C.

6.2.5. Determinations

6.2.5.1. Energy density computation

The power density (P_v, W/m^3) transferred from the ultrasound probe to the sample was determined calorimetrically by using eq. 2.2 reported in paragraph 2.2.5 (pag. 23).

The energy density (MJ/m^3) was estimated by using eq. 2.3 reported in paragraph 2.2.5 (pag. 23).

6.2.5.2. *Lycopene concentration*

The lycopene extraction was performed following the procedure of Sadler, Davis and Dezma (1990), with minor modification, under subdued light to prevent carotenoid degradation and isomerisation. 25 mL of extraction solution (hexane:acetone:ethanol, 2:1:1 v/v/v) was added to 1 g of tomato juice. The mixture was stirred at room temperature for 20 min. Reagent grade water (7.5 mL) was added and stirring was continued for 10 min. The hexane phase, containing lycopene, was separated from the polar phase using a separation funnel. Immediately after extraction, the absorbance of lycopene was measured at 472 nm with a spectrophotometer (Thermo Fischer Scientific, Genesys 10S UV/VIS Spectrophotomer) using hexane as reference. The total lycopene concentration was calculated using the Beer-Lambert law, considering the extinction coefficient of lycopene in hexane equal to $1.8 \cdot 10^5$ L/mol cm.

6.2.5.3. Total phenolic concentration

The total quantity of phenolic components was measured by the Folin-Ciocalteau method (Singleton & Rossi, 1965). 10 mL of methanol:water (1:1 v/v) was added to 1 g of tomato juice and the mixture was stirred for 5 min. The solution was filtered (QL10, size 150 mm) and 100 µL was added to 5 ml of a 1:10 dilution of Folin-Ciocalteau reagents and 0.9 mL of distilled water. After 5 min, 3.5 mL of Na_2CO_3 (115 g/L) was added and the mixture left in the dark, at room temperature for 2 hours. The absorbance of the solution was measured at 765 nm. The optical density was compared to a standard curve prepared with 0 to 500 mg/L of gallic acid and the results were expressed as mg GAE (gallic acid equivalents)/100 g.

6.2.5.4. Light microscopy

Light micrographs were taken using the same equipment reported in paragraph 3.2.5.9 (pag. 36).

6.2.5.5. *Antioxidant activity*

The antioxidant activity was determined following the procedure of Benzie and Strain (1996) determined using ferric reducing antioxidant potential (FRAP). 20 mL of acetone:water (80:20 v/v) mixture was added to 1 g of tomato juice and the mixture was stirred for 5 min. 90 µL of this mixture was added to 3 mL of FRAP solution and incubated in a water bath at 37 °C for 4 min followed by measurement of absorbance at 593 nm against a blank. The optical density was compared to the standard curve for ferrous sulphate ($FeSO_4$) solution, with concentrations between 0 and 1 mM. Results were expressed as FeII (mM) produced/100 g sample.

6.2.5.6. α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of tomato juice was determined spectrophotometrically (UV-2501PC, UV-Vis recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) following the method of Sing et al. (2014) with some modifications. The tomato juice was first centrifuged at 2200 g for 3 min at 20 °C. Aliquots of 30 µL of 1 U/mL α-glucosidase in phosphate buffer (100 mM, pH 7.0) were introduced into 1 mL capacity cuvette in the presence of 100 µL of tomato extract and a volume of 100 mM phosphate buffer (pH 7.0), giving a final volume of 900 µL in the cuvette, and mixed thoroughly. After 10 minutes of incubation at 37 °C, the reaction was started by the addition of 100 µL of 5 mM 4-nitrophenyl- α-D-glucopyranoside (pNGP) solution (Sigma-Aldrich, Milano, Italy) in 100 mM phosphate buffer (pH 7.0) as substrate. The release of p-nitrophenol from pNGP was monitored at 405 nm for 15 min at 37 °C. The changes in absorbance per min were calculated by linear regression, applying the pseudo zero order kinetic model. The eventual stationary phase was excluded from the regression of data. Control samples were run in the absence of tomato extract. The inhibitory activity (*IA%*) of samples on α-glucosidase was calculated according to the following equation:

$$\mathrm{IA\%} = 100 - \left(\frac{k_s}{k_c} \cdot 100\right) \qquad \text{(eq. 6.1)}$$

where k_s and k_c are the constant rates (Abs/min) of the enzymatic activity in the presence or in the absence of the inhibitor.

6.2.6. Data analysis

The results reported here are the average of at least two measurements carried out on two replicated experiments (n ≥ 4). Data are reported as mean value ± standard deviation. Statistical analysis was performed using R v. 2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p<0.05$).

6.3. Results and discussion

6.3.1. Effect of US treatments on lycopene and total phenolic concentration of tomato juice

Table 6.1 shows lycopene and phenolic concentration of tomato juice subjected to high frequency US treatments (378 and 584 kHz) with increasing energy density. No differences in lycopene concentration were found among the untreated and ultrasonically treated samples, regardless of the frequency and energy density. The total phenolic concentration was slightly affected by the application of high frequency US treatments. A significant increase of the phenolic concentration was observed for the tomato juice subjected to ultrasounds at 378 kHz, providing an energy density of 250 MJ/m^3. However, from a practical point of view, this result does not appear to be relevant in terms of total phenolics recovery. To our knowledge, only Golmohamadi, et al. (2013) have investigated the effect of high frequency US treatments (490 kHz) on the total phenolics in red raspberry puree. They found that ultrasonication did not affect the total phenolic concentration. However, results of Golmohamadi and co-workers cannot be directly compared with those obtained in this study, due to discrepancies in the frequencies as well as the lack of information concerning the energy density applied.

Levels of lycopene and total phenolics presented in the tomato juice after treatment at 378 and 583 kHz (250 MJ/m^3) were compared with those obtained at 24 kHz with the same energy density (Table 6.1). In fact, although the application of low frequency US treatments is commonly used in food processing, no data are present in the literature comparing the effect of high and low frequency ultrasounds at the same energy density. It can also be observed that the ultrasound treatment at 24 kHz did not cause any significant change in lycopene concentration. This result is in agreement with previously published data (Anese et al., 2013) relating to tomato juice ultrasonically treated at 24 kHz with an energy density of 731 MJ/m^3. Moreover, the low frequency US treatment did not significantly modify the total phenolic concentration of tomato juice. This result is, however, contrary to that of Chemat et al. (2011) and the discrepancy may be attributed to differences in the process parameters.

Table 6.1. Lycopene and total phenolic concentrations, antioxidant activity and α-glucosidase inhibitory activity of untreated and ultrasonically treated tomato juice. Data are referred to increasing energy density provided at 24 kHz, 378 kHz and 583 kHz.

Frequency (kHz)	Energy density (MJ/m^3)	Lycopene (mg/g)	Total phenolic (GAE mg/100 g)	Antioxidant activity (FeII mM/100 g)	α-glucosidase inhibitory activity (%)
Untreated	0	0.35 ± 0.01^{a}	192.1 ± 4.0^{bc}	25.0 ± 2.7^{a}	72 ± 4^{a}
24	250	0.36 ± 0.01^{a}	193.9 ± 1.0^{bc}	n.d.	74 ± 2^{a}
378	15	0.35 ± 0.01^{a}	192.9 ± 1.0^{bc}	17.8 ± 0.5^{b}	n.d.
	59	0.35 ± 0.02^{a}	191.4 ± 1.0^{bc}	15.7 ± 0.3^{b}	78 ± 2^{a}
	62	0.36 ± 0.01^{a}	205.0 ± 2.0^{ab}	19.2 ± 0.5^{b}	n.d.
	250	0.37 ± 0.02^{a}	212.2 ± 6.0^{a}	18.8 ± 1.1^{b}	78 ± 2^{a}
583	8	0.35 ± 0.00^{a}	197.1 ± 3.0^{bc}	18.7 ± 0.6^{b}	n.d.
	31	0.35 ± 0.01^{a}	195.0 ± 2.0^{bc}	17.8 ± 2.2^{b}	76 ± 1^{a}
	62	0.33 ± 0.00^{a}	194.3 ± 3.0^{bc}	19.3 ± 1.8^{b}	n.d.
	250	0.35 ± 0.01^{a}	187.1 ± 5.0^{c}	18.7 ± 0.3^{b}	79 ± 3^{a}

Means with different letters within the same column are significantly different ($p<0.05$).
n.d.:not determined.

Although the application of high and low frequency ultrasounds did not affect the levels of bioactive components, slight differences in the tomato microstructure were observed. Figure 6.1 shows the micrographs of tomato juice subjected to US treatments at 24 kHz, 378 kHz, 583 kHz at an energy density of 250 MJ/m^3. When compared to the untreated tomato juice, the low frequency ultrasonically processed samples showed a partial disruption of cell membranes with carotenoids distributed into the matrix. However, no differences between the untreated sample and those processed at 387 and 583 kHz were observed. The differences in the microstructure of samples treated at low and high frequency can be attributed to cavitation phenomena occurring during US treatment. In fact, during low frequency (24 kHz) US treatments, transient cavitation phenomena are responsible for the rapid change in fluid pressure and temperature, which might cause cell wall disruption. By contrast, microstreaming phenomena are generated by stable cavitation at high frequency (378 kHz and 583 kHz) US treatments; these are reported not to cause dramatic structure changes (McClements, 1995).

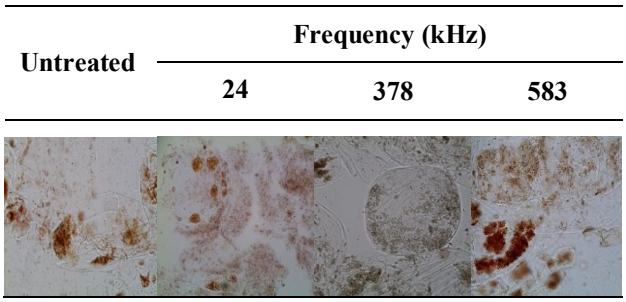


Figure 6.1. Images of tomato juice subjected to ultrasounds at 24 kHz, 378 kHz and 583 kHz (at 250 MJ/m^3) along with an untreated sample.

6.3.2. Effect of US treatments on antioxidant properties and α-glucosidase inhibitory activity of tomato juice

Table 6.1 shows the effect of high frequency US treatments at 378 kHz and 583 kHz on the antioxidant activity of tomato juice. As compared with the untreated sample, the application of the high frequency ultrasounds caused a significant reduction in the antioxidant activity. This seems to be in contradiction with the levels of lycopene and total phenolic compounds which were affected by ultrasound. It should be kept in mind that these are not the only compounds with antioxidant properties present in tomato product. For instance, ascorbic acid is a well known antioxidant and this vitamin has been destroyed by high frequency ultrasounds (Golmohamadi et al., 2013; Portenlänger & Heunsiger, 1992) This result is in contrast with data reported by Ashokkumar et al. (2008) for a phenol model aqueous solution subjected to 358 kHz. The authors attributed the increase in antioxidant properties of the phenolic component in the model solution to the generation of $OH^{\cdot}$ radicals due to the homolysis of water molecules and subsequent phenol hydroxylation. The decrease in antioxidant capacity observed under our

experimental conditions may be attributed to the addition of the hydroxyl radicals in a non-preferred position of the aromatic ring. This has previously been observed for a cyanidin 3-glucoside model system (Ashokkumar et al., 2008). These results suggest that controlled hydroxylation is needed to promote an increase in the antioxidant properties of phenolic compounds, which is indeed difficult to achieve. Increasing attention has been recently paid towards the capability of phenolic compounds to control blood glucose levels related to type 2 diabetes (Hanhineva et al., 2010; Lordan et al., 2013). In particular, phenolic compounds were found to inhibit digestive enzymes involved in starch breakdown, such as α-glucosidase. Table 6.1 shows the α-glucosidase inhibitory activity of tomato juice subjected (or not) to ultrasounds at 24 kHz, 378 kHz and 583 kHz at an energy density of 250 MJ/m^3. It can be observed that in our experimental conditions for the untreated sample α-glucosidase inhibition was about 72%. Moreover, no significant changes in α-glucosidase inhibition were found when considering the US treated samples.

6.4. Conclusion

The results of this study showed that US treatments at 24, 378 and 583 kHz had no effect on major bioactive compounds (i.e. lycopene and total phenolic compounds) as well as on α-glucosidase inhibitory activity of tomato juice regardless the frequency and the energy density. On the other hand, the antioxidant properties appear to be reduced by high frequency ultrasounds. Thus, we conclude that high frequency US treatments do not seem to effectively alter the bioactive concentration or improve the functionality of tomato juice.

Chapter 7

Effect of low frequency US treatment on lycopene stability and bioaccessibility in tomato juice

Low frequency US (18-40 kHz) treatments are largely investigated in the food sector. In particular, several studies showed that US treatments can induce structural and functional modifications of macromolecules (i.e. proteins and polysaccharides) (Ashokkumar et al., 2008; Vercet et al., 2002; Wu et al., 2008).
Tomato is a worldwide important crop due to its large consumption, versatility and its high lycopene content. The high degree of conjugation and hydrophobicity confer to lycopene molecule unique biological properties, including strong antioxidant activity (Shi & Le Maguer, 2000). It has been suggested that a lower risk of developing cardiovascular diseases and cancer following a diet rich in this carotenoid might be related to lycopene antioxidant properties (Tanaka, Shnimizu, & Moriwaki, 2012). These effects are strictly related to the carotenoid bioaccessibility, i.e. the fraction of a nutrient that is released from the food matrix and incorporated into micelles during digestion before being absorbed by enterocytes (Hedrén, Diaz, & Svanberg, 2002). Recently, Anese et al. (2013) investigated the effect of increasing ultrasound energies on tomato juice microstructure and lycopene *in vitro* bioaccessibility. These treatments, while causing loss of tomato cells integrity, induced reorganization of partially depolymerised pectins to form a stronger network where lycopene would be entrapped, being thus less accessible for digestion. Next to physical modifications, carotenoids bioaccessbility is strongly affected by the presence of dietary lipids, that would favour its incorporation into micelles (Stahl & Sies, 1992; Colle et al., 2012). However, no data have been found on the effect of US treatments on carotenoids bioaccessbility in tomato added with a lipid phase.

7.1. Aim of the study

The aim of the present study was to investigate the effect of US processing on tomato juice added or not added with a lipid phase on lycopene concentration and *in vitro* bioaccessibility at time zero and during storage under refrigerated conditions. Data were compared with those of analogous samples that were not subjected to ultrasound treatment. Contextually, the changes of viscosity, tomato colour and oxidative status of the lipid fraction of the control and ultrasonically processed samples were studied.

7.2. Materials and methods

7.2.1. Sample preparation

Commercial pasteurized tomato juice was sieved (20 mesh) to separate seeds and coarse particles, and submitted to ultrasound treatment. Tomato juice not subjected to ultrasound treatment (untreated sample) was taken as a control. Aliquots of the unprocessed and processed tomato juice were added with increasing amounts (i.e. 0%, 2.5%, 5% and 10% w/w) of commercial sunflower oil. Aliquots of 40 g of samples were

introduced into 60 mL capacity plastic vessel with pressure lid and stored at 5 °C for up to 100 days. To inhibit microbial growth during storage, 1.5 g/L potassium sorbate and sodium benzoate (Carlo Erba, Milano, Italy) were added to samples.

7.2.2. Low frequency US treatment

Samples were subjected to US treatment using the same equipment reported in paragraph 2.2.3.2 (pag. 22). Aliquots of 60 g of tomato juice were introduced into 250 mL capacity (90 mm height, 75 mm diameter) glass vessel. The horn was placed in the centre of the vessel, with an immersion depth in the fluid of 5 mm. In order to minimise water evaporation during sonication, the vessel was closed with a plexiglas lid fitted with holes allowing horn and thermocouple probes to be placed at the desired positions in the tomato juice. During the ultrasound treatment, tomato juice was kept under stirring to allow temperature to equilibrate within the sample. The temperature was recorded as a function of time using a copper-constantan thermocouple probe (Ellab, Denmark), connected to a data-logger (CHY 502A1, Tersid, Milano, Italy). Treatments were performed for 30 min at an ultrasound frequency and amplitude of 24 kHz and 100 μm, respectively. The effective acoustic power applied during sonication, determined calorimetrically by recording the temperature increase against the time of ultrasound application (Raso et al., 1999), was equal to 71 W, bringing forth to an acoustic energy density of 1462 J/cm^3. The latter was calculated by dividing the acoustic power by the sample volume and multiplying it by the treatment time.

7.2.3. Determination of lycopene concentration

The extraction of lycopene was performed following the procedure of Sadler et al. (1990), with minor modifications. The analysis was carried out under subdued light to prevent carotenoid degradation and isomerisation. 0.5 g NaCl and 50 mL extraction solution (pentane:acetone:ethanol, 2:1:1 v/v/v) were added to 2 g of tomato juice or supernatant containing micelles. The mixture was stirred at room temperature for 20 min. Reagent grade water (15 mL) was added and stirring was continued for 10 min. The apolar phase, containing lycopene, was collected, filtered (Chromafil PET filters, Düren, Germany; 0.20 μm pore size, 25 mm diameter) and transferred to an amber HPLC vial. The HPLC analyses were performed on a Varian Pro Star (model 230, Varian Associates Ldt., Walnut Creek, CA, USA) equipped with a Varian Pro Star photodiode array detector (model 330, Varian Associates Ldt., Walnut Creek, CA, USA), according to Cucu et al. (2012) with some modifications. Lycopene and its isomers were separated at 35 °C on a reversed phase C_{30} column (3 μm×150 mm×4.6 mm, YMC Europe, Dinslaken, Germany) with methanol:2-propanol:tetrahydrofuran (4:3:3 v/v/v) containing 0.05% triethylamine as mobile phase. The flow rate was 1 mL/min and the injection volume 20 μL. Lycopene and its isomers were detected at 472 nm. Retention time and absorption spectra of pure standard (Sigma-Aldrich, Milan, Italy) were used to identify and quantify all-*trans* lycopene. All-*trans* lycopene concentration was expressed as mg/g tomato juice dry matter. Changes in all-*trans* lycopene concentration during storage were expressed as the percentage ratio between the concentration of the all-*trans* lycopene at the time of analysis (C_t) and the concentration of the all-*trans* lycopene at time zero (C_0).

Changes in unidentified lycopene *cis* isomers relative peak area were expressed as the percentage of the all-*trans* lycopene ($A_{all\text{-}trans}$) and *cis* isomers (A_{cis}) total peak area.

7.2.4. In vitro *bioaccessibility*

The lycopene *in vitro* bioaccessibility was measured by simulating human digestion in the stomach and small intestine *in vitro*. The procedure described by Moelants et al. (2012), based on Hedrén et al. (2002), was followed. In particular, 5 g tomato juice was weighed into a 50 mL capacity opaque falcon tube. The sample was diluted with 5 mL NaCl/ascorbic acid solution (0.9% NaCl, 1% ascorbic acid in water), 5 mL stomach electrolyte solution (0.30% NaCl, 0.11% KCl, 0.15% $CaCl_2 \cdot 2H_2O$, 0.05% $KHPO_4$, 0.07% $MgCl_2 \cdot 6H_2O$ in water) and 10 mL of freshly prepared oil-in-water emulsion. The latter was obtained by suspending 1% (w/v) L-α-phosphatidylcholine from egg yolk (Sigma) in water. 5% (v/v) extra virgin olive oil was then added and the mixture was stirred (Polytron, PT 3000, Cinematica, Littau, Swiss) at 9500 rpm during 10 min. Homogenization was performed at 100 MPa for one cycle using a high pressure homogeniser (Panda PLUS 2000, Gea Niro Soavi, Parma, Italy). To simulate the first phase of gastric digestion, the pH of the mixture was adjusted to 4 ± 0.05 with 1 M HCl or 1 M $NaHCO_3$ and 5 mL pepsin solution (0.52% porcine pepsin, from Sigma, in electrolyte solution) was added. After flushing the headspace of the samples with nitrogen for 10 s, the mixture was incubated at 37 °C for 30 min while shaking end-over-end. The pH of the mixture was then acidified to 2 ± 0.05 to mimic the drop of the gastric pH after the intake of a meal (Tyssandier et al., 2003). The headspace of the samples was flushed again with nitrogen for 10 s and the incubation at 37 °C continued for further 30 min. To imitate the passage through the small intestine, the pH of the partially digested tomato product was raised to 6.9 ± 0.05 and 6 mL pancreatin, lipase and bile salts solution (0.4% porcine pancreatin, 0.2% porcine pancreas lipase, 2.5% bile extract, 0.5% pyrogallol, and 1% α-tocopherol, from Sigma, in water) was added. Finally, the headspace of the sample was flushed with nitrogen for 10 s and incubated for 2 h at 37 °C. The digest was centrifuged (XL-70 Ultracentrifuge, Beckman, Palo Alto, CA, USA) at 165000 g for 67 min at 4 °C to separate the micelles. The supernatant was collected, filtered (Chromafil PET filters, Düren, Germany; 0.20 μm pore size, 25 mm diameter) and analysed for lycopene content. The lycopene *in vitro* bioaccessibility was defined as the percentage ratio between the all-*trans* lycopene concentration in the micelles at the time of the analysis (B_t) and the all-*trans* lycopene concentration in the sample at time zero (C_0). Changes in all-*trans* lycopene *in vitro* bioaccessibility during storage were expressed as the percentage ratio of lycopene bioaccessibility measured at the different storage times (% B_t/C_0) and at time zero (% B_0/C_0).

7.2.5. Viscosity

Oscillatory measurements were carried out in the frequency range of 0.1-10 Hz, at a constant stress amplitude of 0.4 Pa (i.e. in the linear viscoelastic region of the material) and 20 °C, by using a Stresstech Rheometer (ReoLogica Instruments AB, Lund, Sweden) equipped with a concentric cylinder geometry (C25).

7.2.6. Total solids content

The total solids content was measured by gravimetric method (AOAC, 1995).

7.2.7. Colour

Colour analysis was carried out using the same equipment reported in paragraph 3.2.5.3 (pag. 35). Colour was expressed in L*, a* and b* scale parameters and a* and b* were used to compute the hue angle ($\tan^{-1}$ b*/a*). An increase of this colour parameter was used as an index of redness loss.

7.2.8. Peroxide value

The peroxide value (PV) of the samples was assessed according to the European Official Methods of Analysis (1991).

7.2.9. Light microscopy

Light micrographs were taken using the same equipment reported in paragraph 3.2.5.9 (pag. 36).

3.2.10. Data analysis

Statistical analysis was performed using STATISTICA (5.1, Statsoft Inc., Cary, NC, USA). Bartlett's test was used to check the homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p<0.05$). Correlation analysis was carried out by using Microsoft Office Excel 2007.

7.3. Results and discussion

7.3.1. Effect of ultrasounds and oil incorporation on lycopene concentration and in vitro *bioaccessibility*

Untreated and ultrasonically treated tomato samples were first characterized for their total solids content and viscosity (Table 7.1). Despite the loss of water as a consequence of the ultrasound treatment was negligible, viscosity greatly increased. The effect of US processing on the structural properties of tomato juice has already been investigated (Anese et al., 2013). US treatment can cause partial de-esterification of pectin molecules, which may subsequently establish hydrogen bonds and hydrophobic interactions, giving rise to a new network, with increased gel-like properties. No changes in the rheological parameter were found during the storage of tomato juice (data not shown), indicating that the present experimental conditions caused a permanent viscosity increase. The light microscope images of the untreated and ultrasonically treated tomato juices (Table 7.1) clearly show differences in cell integrity. In particular, the unprocessed samples

presented intact cells containing lycopene crystals, while broken cells and lycopene distributed in the matrix can be observed in the processed tomato juice.

Table 7.1. Total solids content, viscosity and images of untreated and ultrasonically (US) treated tomato juices.

Sample	Total solids (g/100 g)	Viscosity (Pa s)	Image (200x)
Untreated	8.04 ± 0.03^{a}	$2.7 \pm 1.0^{a'}$	
US treated	8.33 ± 0.02^{a}	$13.6 \pm 1.7^{b'}$	

Significant difference is indicated by different letters ($p<0.05$)

All-*trans* lycopene concentration of freshly prepared untreated and ultrasonically treated tomato juices containing no or 2.5%, 5% and 10% sunflower oil are shown in Table 7.2.

Table 7.2. All-*trans* lycopene concentration (C_0) and bioaccessibility (% B_0/C_0) of untreated and ultrasonically (US) treated tomato juices containing no or increasing amounts of sunflower oil.

Oil (% w/w)	All-*trans* lycopene (mg/g dm)		Lycopene bioaccessibility (%)	
	Untreated	US treated	Untreated	US treated
0	1.95 ± 0.36^{a}	1.51 ± 0.28^{a}	1.06 ± 0.27^{ab}	1.24 ± 0.36^{a}
2.5	1.44 ± 0.05^{a}	1.64 ± 0.10^{a}	0.99 ± 0.30^{ab}	0.85 ± 0.17^{bd}
5.0	1.42 ± 0.11^{a}	1.47 ± 0.05^{a}	0.33 ± 0.05^{c}	0.84 ± 0.15^{bd}
10.0	1.58 ± 0.12^{a}	1.31 ± 0.08^{a}	0.35 ± 0.07^{cd}	0.65 ± 0.05^{d}

Significant difference is indicated by different letters ($p<0.05$)

Lycopene concentrations were in the range of those reported in the literature data (Tonucci et al., 1995). The addition of oil did not cause any change in the all-*trans* lycopene concentration. Moreover, no significant differences in the carotenoid content were found between untreated and ultrasonically treated samples containing a same amount of oil. These results are in agreement with those already described in the literature for tomato derivatives subjected to US and high pressure homogenization treatments associated to a temperature increase not exceeding 100 °C (Anese et al., 2013; Colle et al., 2010a; Knockaert et al., 2012; Perez-Conesa et al., 2009). It is

noteworthy that under the present experimental conditions temperature never exceeded 90 °C.

Table 7.2 also shows the lycopene *in vitro* bioaccessibility at time zero of the untreated and ultrasonically treated tomato juices containing no or 2.5%, 5% and 10% sunflower oil. Except for the 5% oil-containing samples, no significant differences in lycopene *in vitro* bioaccessibility were found between the untreated and ultrasonically processed samples having the same oil content, in contrast with data from the literature (Anese et al., 2013; Colle et al., 2010b; Panozzo et al., 2013). These authors reported a decrease in lycopene *in vitro* bioaccessibility consequently to ultrasound or high pressure homogenization processing of tomato juice. In fact, despite these processes favoured lycopene release from tomato cells, its uptake into the micelles was hindered by the formation of a strong fibre network entrapping the carotenoid. Further on, the lycopene bioaccessibility values relevant to the samples with no oil added were approximately two to four fold higher than those found by Anese et al. (2013) for tomato juice subjected to similar processes. These discrepancies can be due to differences in the methods used to assess the carotenoid *in vitro* bioaccessibility. In fact, differently from what reported in the aforementioned papers, the lycopene bioaccessibility in tomato juices in this study was determined in the presence of an oil-in-water emulsion, added just before the *in vitro* digestion, together with a lipase containing solution (Moelants et al., 2012). The oil-in-water emulsion was added to better mimic the emulsification process in the stomach during lipid digestion (Carey, Small, & Bliss, 1983). By emulsifying, the surface area of the emulsion would increase, thus favouring lycopene extraction mainly from the phospholipid-rich chromoplasts (Lenucci et al., 2012) and its incorporation into the oil droplets. The lipid droplets are formed by a hydrophobic core containing triglycerides, lycopene and other fat soluble molecules, and surrounded by an amphipathic surface monolayer (Bauer, Jakob, & Mosenthin, 2005). Hydrolysis at the oil droplet surface by lipase would then allow the lycopene to be released and subsequently incorporated into the bile salt micelles (Carey et al., 1983). To confirm this hypothesis, lycopene *in vitro* bioaccessibility was also assessed in untreated and ultrasonically treated tomato juices in the absence of the oil-in-water emulsion. In both the cases, the lycopene bioaccessibility values were similar to those reported in the previous study (Anese et al., 2013) and approximately 60% lower than those attained for the emulsion-added counterparts. Similar results are reported by Moelants et al. (2012) for β-carotene bioaccessibility measured in carrot-derived suspension without oil addition, with the addition of 2% olive oil as such and with the addition of 2% oil-in-water emulsion at the start of the *in vitro* digestion procedure. The authors found that emulsion addition led to the greatest increase in carotenoid uptake into the micellar phase, followed by the olive oil alone. Overall, the use of the oil-in-water emulsion in the digestion procedure would explain not only the higher lycopene bioaccessibility values we found in this work as compared to the already published ones, but also the almost negligible differences between the untreated and ultrasonically processed tomato juices. It can be inferred that the use of the oil-in-water emulsion could improve the lycopene transfer into the micelles from the ultrasonically processed matrix, where the dispersed carotenoid is

tightly entrapped (Table 7.1).
Table 7.2 also shows that the *in vitro* bioaccessibility of lycopene significantly decreased with the increase of the oil content in both the untreated and ultrasonically treated tomato juices, in agreement with data of Colle et al. (2012). These authors reported that, although lycopene bioaccessibility may be improved by the presence of fat, high levels of lipids containing a large fraction of long chain triglycerides (e.g. olive oil, sunflower oil and fish oil) significantly decreased the lycopene bioaccessibility (Huo et al., 2007). In fact, an increase of the lipid amount could be responsible for an incomplete hydrolysis of triglycerides (Porter et al., 2004). It must be pointed out that, in our experimental conditions, the addition of the oil-in-water emulsion at the start of the *in vitro* digestion procedure contributed to increase the lipid load.

7.3.2. Effect of ultrasounds and oil incorporation on lycopene concentration and in vitro bioaccessibility during storage

Figure 7.1 shows the changes in all-*trans* lycopene concentration and *cis* isomers of untreated and ultrasonically treated tomato juices containing no or 10% sunflower oil during storage at 5 °C. The highest oil amount was chosen to better show the effect of concentration. No significant differences in the all-*trans* lycopene levels among the samples were found at a same storage time ($p>0.05$). Moreover, lycopene concentration did not vary in the first 15 days of storage, while it significantly decreased up to 30 days ($p<0.05$). By prolonging the storage time, no further decrease in lycopene concentration was observed. Similarly, no significant differences of the relative *cis* isomers peak area values were found among the samples at a same storage time ($p>0.05$). On average, initially only 5% ± 1 of lycopene was present as unidentified *cis* isomers, which is consistent with the thermodynamic stability of the all-*trans* form (Shi & Le Maguer, 2000). The relative peak area of lycopene *cis* isomers increased after 60 days of storage, reaching a mean value of 10% ± 1 at 100 days. These results suggest that the ultrasound treatment as well as the presence of oil slightly affected lycopene isomerization, in agreement with other findings showing that the relative concentration of lycopene *cis* isomers did not vary significantly when tomato is exposed to mild process temperature (Nguyen & Schwartz, 1998).

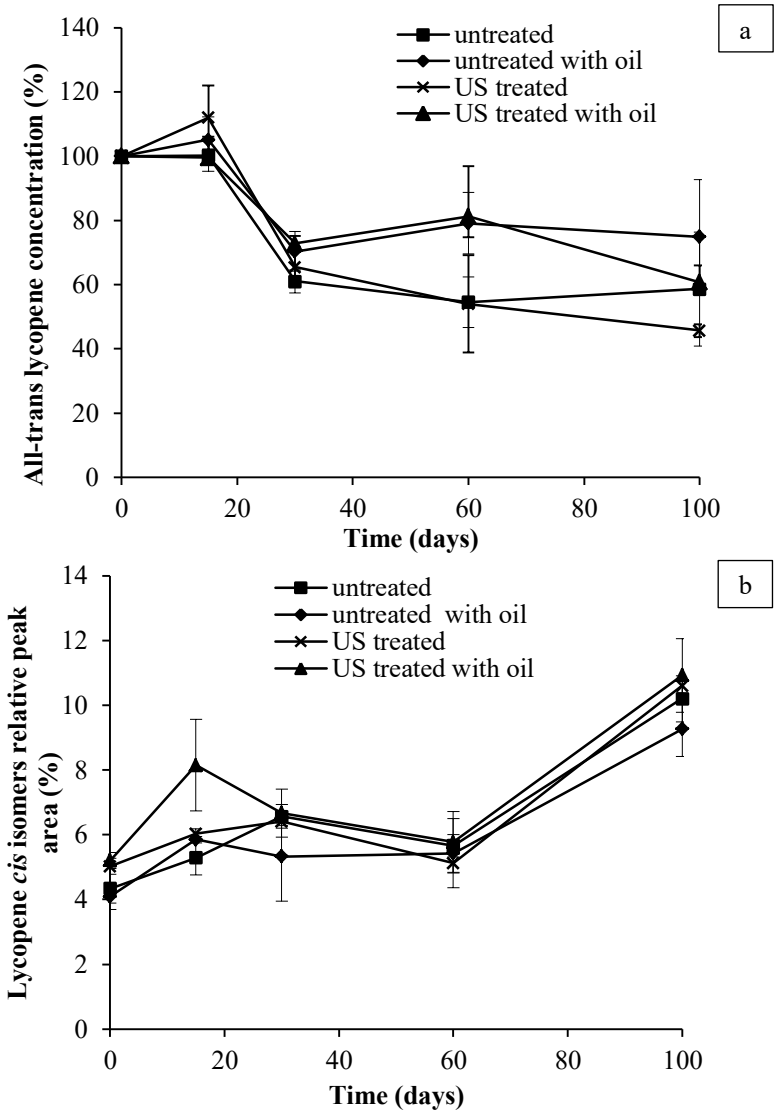


Figure 7.1. Relative all-*trans* lycopene concentration (% C_t/C_0) (a) and lycopene *cis* isomers relative peak area (% $A_{cis}/A_{all\text{-}trans}$) (b) of untreated and ultrasonically (US) treated tomato juices containing no or 10% sunflower oil during storage at 5 °C.

Figure 7.2 shows the changes of the lycopene *in vitro* bioaccessibility of untreated and ultrasonically treated tomato juices containing no or 10% sunflower oil during storage at 5 °C. After an initial lag period (the differences between samples at time zero and after 15 days storage were not significantly different), the lycopene *in vitro* bioaccessibility significantly decreased up to 60 days of storage, whereas, by prolonging the time, only slight changes of this parameter occurred. The reduction of lycopene *in vitro* bioaccessibility ranged between 50 and 80%, the untreated tomato juices showing a greater decrease than the ultrasonically treated ones. A protective effect of the highly

viscous matrix of the ultrasonically treated tomato juice towards lycopene could explain the lower decrease in the *in vitro* bioaccessibility of this sample during storage as compared to the unprocessed counterpart.

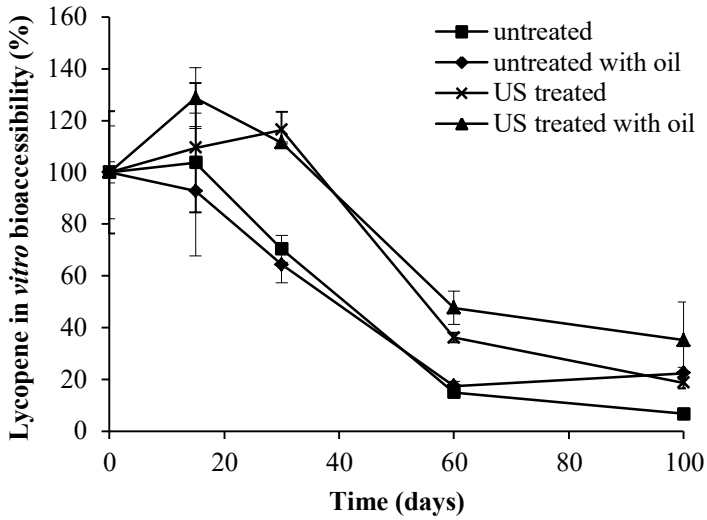


Figure 7.2. Changes in lycopene *in vitro* bioaccessibility (% B_t/B_0) of untreated and ultrasonically (US) treated tomato juices containing no or 10% sunflower oil during storage at 5 °C.

An evidence of lycopene degradation in the untreated and ultrasonically treated tomato juices containing no or 10% sunflower oil during storage is given by the changes of hue angle values (Figure 7.3). After a 15 days lag time, the values of this colour parameter progressively increased during storage, indicating a redness loss. The non-containing oil samples subjected or not to the ultrasound treatment showed the lowest hue angle values. Bleaching was greater in the ultrasonically treated tomato juice containing oil, followed by the untreated sample added with oil. These results are consistent with the peroxide values of the lipid fraction of the untreated and ultrasonically treated tomato juices containing oil (Figure 7.4). Initially, a lag phase of about 30 days was observed. It can be inferred that the naturally occurring carotenoids might protect the lipid fraction from oxidative reactions by virtue of their strong antioxidant activity (Anese et al., 2002). As known, the protective action exerted by lycopene may result in redness loss. After this time, although a marked increase in peroxide values was observed for both samples, the rate of formation was greater in the ultrasonically processed tomato juice, plausibly due to the contribution of radical species generated as a consequence of the acoustic cavitation (Ashokkumar et al., 2008) as well as loss of ascorbic acid protective action, due to its ultrasonically induced degradation (Adekunte et al., 2010).

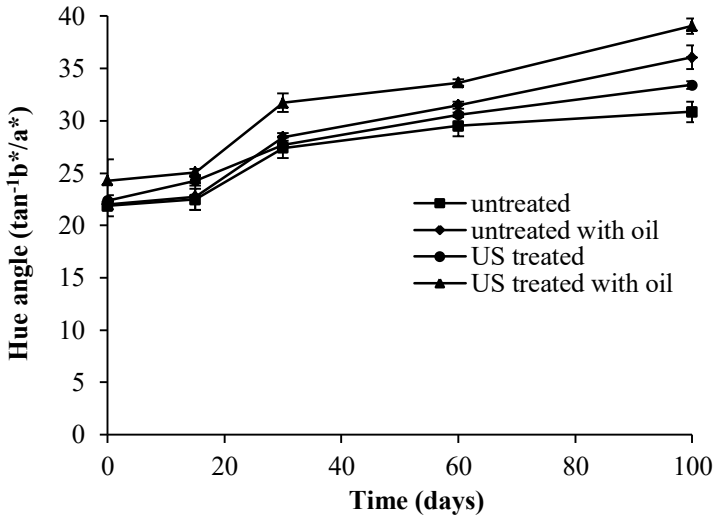


Figure 7.3. Hue angle of untreated and ultrasonically (US) treated tomato juices with no or 10% sunflower oil during storage at 5 °C.

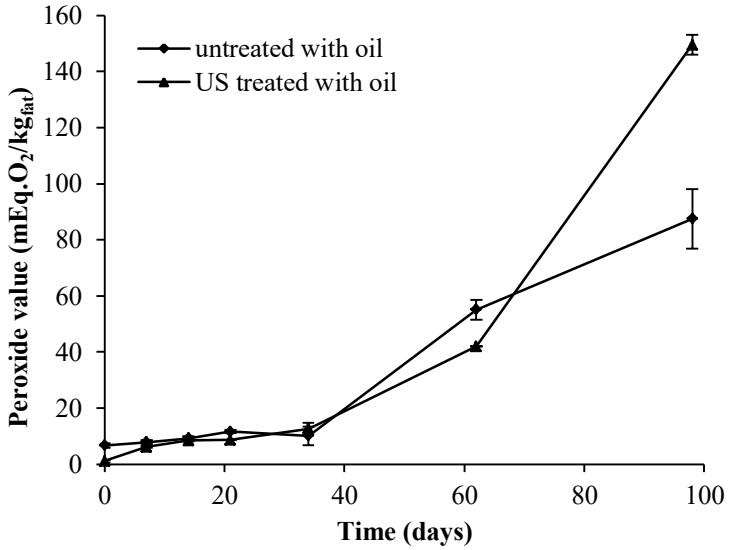


Figure 7.4. Peroxide value of untreated and ultrasonically (US) treated tomato juices containing 10% sunflower oil during storage at 5 °C.

Actually, a good positive correlation was found between the colour and peroxide values data ($R=0.85$, $p<0.01$) of the untreated and ultrasonically treated tomato juices containing oil. The hue angle parameter correlated well also with the lycopene concentration ($R=0.74$, $p<0.01$) and *in vitro* bioaccessibility ($R=0.74$, $p<0.01$). Overall these results suggest that the losses of lycopene concentration and bioaccessibility occurring during storage may be related to an increase in carotenoid susceptibility to degradation in the presence of unsaturated lipids (i.e. sunflower oil). In fact, carotenoid oxidation reactions are favoured by co-oxidation with lipid hydroperoxides (Rodriguez-Amaya, 2001). However, this may be not the only mechanism for lycopene *in vitro* bioaccessibility reduction. As the decrease of lycopene bioaccessibility during storage was greater than that of lycopene levels, it might be suggested that, in addition to lycopene degradation, other factors, whose nature has to be clarified, could contribute to reduce the lycopene *in vitro* bioaccessibility.

7.4. Conclusions

As already mentioned, the results of a number of recent studies have demonstrated that innovative technologies, such as those based on high pressure, ultraviolet light and ultrasound, if properly applied, can contribute to obtain tailored ingredients and semi-manufactured products, i.e. technologically evolved ingredients and intermediate products, able to accomplish specific functions. This work was addressed to explore the possibility to modify the technological and nutritional functionality of tomato juice by using ultrasound processing.

The results reported here clearly show that ultrasound processing of tomato juice, while causing a great increase in viscosity, only slightly affected all-*trans* lycopene concentration and *in vitro* bioaccessibility. However, dietary oil incorporation to either the untreated or ultrasonically treated tomato juice caused a decrease in lycopene bioaccessibility.

Upon storage, after an initial lag period, the lycopene *in vitro* bioaccessibility of tomato juices containing no or 10% oil greatly decreased, mainly due to carotenoid degradation.

It can be concluded that ultrasound treatments can be actually applied to steer the structure of tomato derivatives without impairing their stability and functionality. However, these properties can be negatively affected by dietary oil incorporation and storage.

Chapter 8

Effect of PEF treatment on carotenoids concentration and bioaccessibility in tomato fractions

Several studies relate a high intake of bioactive compounds present in fruit and vegetables to human health benefits. Among the large spectrum of bioactive compounds, lycopene and β-carotene, play an important role in human health because of their powerful antioxidant properties and pro-vitamin A activity. To study the carotenoids health related functions, their bioavailability needs to be evaluated. However, the bioavailability is strongly related to their bioaccessibility, that is the fraction released from the matrix and available for the intestinal absorption (Holst & Williamson, 2008; Parada & Aguilera, 2007). The specific localization of carotenoids into the chromoplasts as well as the structural barriers within the cell govern carotenoids bioaccessibility. In particular, chromoplast and cell membrane as well as cell wall are the limiting factors for both β-carotene and lycopene bioaccessibility in tomato and carrot (Jeffery, Holzenburg, & King, 2012; Palmero et al., 2013; Schweiggert et al., 2012).

Several studies investigated the effect of thermal treatment, high pressure homogenization or high power ultrasounds on carotenoids bioaccessibility in tomato juice (Anese et al., 2013; Colle et al., 2010a; Colle et al., 2010b; Panozzo et al., 2013). However, the structural complexity of the tomato matrix did not allow to disentangle the effect of the various processes on the different cell barriers thus to understand which are the key factors governing carotenoids bioaccessibility. To tackle this issue, Palmero et al. (2013, 2014, 2016) used tomato fractions posing different physical barriers to carotenoids bioaccessibility (i.e chromoplasts and clusters cells) and applied thermal or high pressure homogenization treatments.

Pulsed electric fields (PEF) is a widely explored technology for inducing the permeabilization of cell membranes. The effect of PEF at low electric fields applied individually or in combination with heating has been investigated in order to improve the extraction yield of intracellular compounds present in fruits and vegetables tissue (Donsì, Ferrari, & Pataro, 2010). However, only a few studies investigated the effect of PEF on the extraction of lipophilic compounds, such as carotenoids (Luengo, Álvarez, & Raso, 2014; Wiktor et al., 2015).

8.1 Aim of the study

The aim of this study was to investigate the effect of PEF on carotenoids bioaccessibility in tomato fractions. To this purpose tomato tissue, cells clusters, single cells and chromoplasts were isolated and subjected to PEF and heating, in combination or individually. Contextually, microstructure, conductivity and carotenoids concentration of untreated and treated samples were determined.

8.2. Materials and methods

8.2.1. Materials

A 30 kg batch of red tomatoes (*Lycopersicum esculentum*) was purchased in a local store in The Netherlands and stored at 7 °C until their use for the experiment. Tomato samples were prepared fresh for every trial from the same batch of fruits to minimize the influence of the matrix.

8.2.2. Experimental set-up

Four fractions with different level of structural barriers corresponding to tissue, cells clusters, single cells and chromoplasts were isolated from tomato and subjected to PEF (PEF), heating (HEAT) or combined PEF and heating (PEF+HEAT) . Microstructure, conductivity, carotenoids concentration and in *vitro* bioaccessibility analyses were performed to each fraction. Figure 8.1 depicts schematically the experimental set-up performed in the present study.

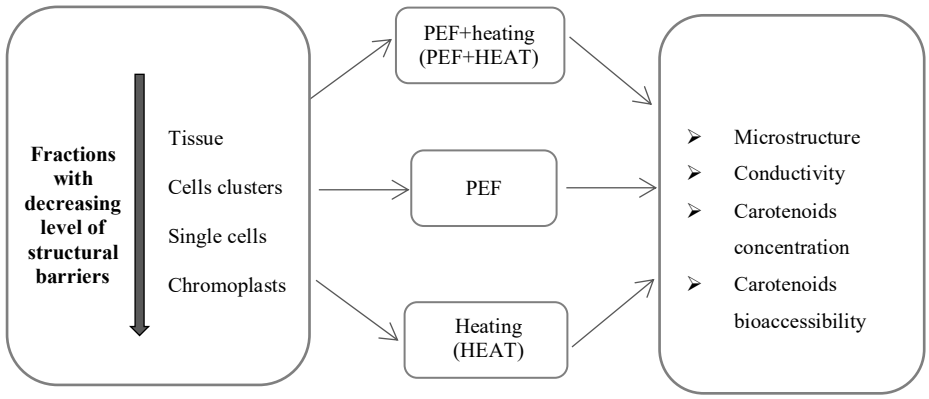


Figure 8.1. Experimental set-up.

8.2.3.Preparation of tomato fractions

8.2.3.1. Tissue

Tomato tissue was prepared by removing skin and placental tissue and cutting the mesocarp into cubes of 0.5 cm length.

8.2.3.2. Cells clusters

Tomato cubes, obtained by previous discard of skin and placental tissue, were blended in a kitchen blender (5 s for 3 times). The cells clusters were obtained by separating the juice with the use of wet sieving equipment (Analysette 3 Spartan, Idar-Oberstein, Germany). The size of the fraction considered in this study ranged between 71-350 µm.

8.2.3.3. Single cells

Single cells were obtained based on the procedure of McAtee, Hallett, Johnston, & Schaffer (2009) with minor modifications. Tomato cubes, obtained by previous discard of skin and placental tissue, were immersed in a 0.05 M Na_2CO_3 in 0.3 M mannitol

solution. The solution was heated at 90 °C for 30 minutes under continuous stirring, and filtered with a sieve (1 mm). The cells were isolated by filtering the solution through a cheesecloth.

8.2.3.4. Chromoplasts

Chromoplasts were isolated based on the procedure of Hansen and Chiu (2005) as described by Palmero et al. (2013). Tomato cubes, obtained by previous discard of skin and placental tissue, were blended in a kitchen blender (5 s for 5 times) with 0.05 M EDTA solution (1:1 ratio). The obtained juice was filtrated using a cheesecloth and the filtrate was centrifuged (Beckman Coulter Avanti J-26XP centrifuge, Palo Alto, CA, USA) at 27250 g and 4 °C for 30 min. The pellet, consisting of the chromoplasts, was re-dissolved in 5 mg/mL NaCl.

8.2.4. Treatments

8.2.4.1.Pulsed electric fields treatments

Pulsed electric fields (PEF) treatment was carried out using a NP110-60 system (IXL Netherlands B.V.) with an output voltage of 3.8 kV. The system provided monopolar, rectangular shaped pulses of average 350 µs width. The treatment chamber consisted of a batch chamber with two circular 316 stainless steel electrodes with a surface area of 28.3 cm^2, resulting in a 56.5 cm^3 total volume. The distance between the electrodes was 2.0 cm. Aliquots of 57 g of tomato fractions were put into the chamber subjected to PEF treatments characterized by a total energy input (Q) (MJ/kg) of 7.6 MJ/kg. The latter was calculated according to Zhang, Barbosa-Cánovas and Swanson (1995) (eq. 8.1), by using the following equation:

$$Q = \frac{V^2 t}{R m} \qquad (eq\ 8.1)$$

where V is the voltage (kV), t is the total treatment time (s), R is the resistance (Ohm) and m is the sample mass (kg). Two PEF treatments were performed: (i) 90 pulses at 1Hz repetition rate in order to reach an initial temperature of 90 °C. Afterwards, 210 pulses where delivered at 0.167 Hz s within 30 minutes at an equilibrium temperature in the range of 85-90 °C. This treatment was indicated as PEF+HEAT; (ii) 600 pulses where delivered at 0.33 Hz at an equilibrium temperature in the range of 40-45 °C. This treatment was indicated as PEF.

The temperature was measured at the end of the treatment by using a copper-constantan thermocouple probe connected to a data logger (YC 727UD, TMS Europe Ltd, Hope Valley, United Kingdom). After the treatments, the samples were cooled in a water-ice bath.

8.2.4.2. Heating

The total temperature–time combination received by the samples during PEF+HEAT was applied to the sample in absence of the electric field. To this purpose, aliquots of 57 g of tomato fractions were heated in a thermostatic water bath (Ika Werke, MST BC, Staufen, Germany) under continuous stirring. The sample reached 90 °C within 5 min and the temperature was maintained constant at 90 °C for 25 min. This treatment was indicated as HEAT. The temperature was recorded using a copper-constantan thermocouple probe connected to a data logger (YC 727UD, TMS Europe Ltd, Hope Valley, United Kingdom). After the treatment, the samples were cooled in a water-ice bath.

8.2.5. Microscopy analysis

Microstructure of tomato fractions was analysed using an optical microscope (Axioskop Zeiss, Göttingen Germany). The pictures were taken by a digital camera (Axiocam HCR, Göttingen, Germany). Tissue, cells clusters and single cells were analysed with 10x lens, while 100x objective lens was used for chromoplasts.

8.2.6. Impedance measurement

Measurement of electrical complex impedance was used to characterized tissue permeabilization after treatments (Donsì, Ferrari, & Pataro, 2010). The measurement was conducted by loading the sample in a test vessel between two 0.3 mm platinum wires separated 1.0 cm and inserted 1.0 cm deep into the sample. The Pt wires were connected to an impedance analyser which consisted of a Rigol DG1022 function generator and a Rigol DS1054Z oscilloscope. The generator produced a sinusoidal voltage of 2 V peak-peak with a frequency ranging between 1 kHz and 1 MHz. All the measurement were carried out at 20 °C. The electrical conductivity is the ratio of the current through the sample and the voltage drop across the sample, multiplied by the cell constant ($1 cm^{-1}$) of the wire electrode set-up. Conductivities, given in the unit S/m, are corrected for evaporation in the heated samples (leading to an increase in ion concentration) by normalizing to the sample weight i.e. conductivity is expressed in units of S/m·g.

8.2.7. Cell disintegration index

Cell disintegration index (Z_p) was computed according to Angersbach, Heinz and Knorr (1999). This index indicate the proportion of permeabilized cells based on the frequency dependence of conductivity of intact and permeabilized plant tissue. The Z_p was calculated by using the following equation (eq. 8.2):

$$Z_p = 1 - \left(\frac{K_h}{K_h'}\right) \cdot \frac{(K_h' - K_l')}{(K_h - K_l)} \qquad (eq.\ 8.2)$$

Where K_h and K_l' are the conductivities of untreated and treated tomato fraction at 1 kHz, respectively, and K_h and K_h' are the electrical conductivities of untreated and

treated tomato fraction at 1 MHz, respectively. The Z_p varies between 0 for intact tissue and 1 for a tissue with all the cells permeabilized.

8.2.8. Carotenoids concentration

The extraction of carotenoids from tomato fractions or micelles was performed following the procedure of Sadler et al. (1990) with minor modifications. The analysis was carried out under subdued light to prevent carotenoid degradation and isomerisation. 0.5 g NaCl and 50 mL extraction solution (hexane:acetone:ethanol, 2:1:1 v/v/v) were added to 2 g of tomato sample or supernatant containing micelle fraction. After 20 min of stirring at room temperature, 15 mL of reagent grade water was added and stirring was continued for 10 min. The apolar phase, containing carotenoids, was collected, filtered (Chromafil PET filters, Düren, Germany; 0.20 µm pore size, 25 mm diameter) and transferred to an amber HPLC vial. The HPLC analyses were performed on a Ultimate 3000 Rapid Separation LC System (Thermo Fisher Scientific, Sunnyvale, CA, USA) equipped with a Ultimate 300 RS photodiode Diode array detector (DAD-3000RS, Thermo Fisher Scientific, Sunnyvale, CA, USA). β-carotene and all-*trans* lycopene were separated at 25 °C on a reversed phase C_{30} column (250 x 4.6 mm, particle size 5 µm YMC Europe, Dinslaken, Germany) with a gradient of two methanol:methyl tert-butyl ether eluents (eluent A; 90:10, eluent B; 10:90) containing 0.1% BHT. The gradient was as follow: 0 min: 88% A, 12% B; 2-4 min: 73% A, 26% B; 4-6 min: 57% A, 43% B; 6-8 min: 20% A, 80% B; 9-14 min: 0% A and 100% B; 15-25 min: 88% A, 12% B. The flow rate was 1.05 mL/min with an injection volume of 20 µL. β-carotene and all-*trans* lycopene were identified based on retention times and spectral characteristics compared to the standards (data not shown). To quantify the carotenoids, HPLC-DAD responses were measured at 450 nm for β-carotene and at 470 nm for all-*trans* lycopene. The carotenoids content was calculated based on their calibration curves and expressed as µg/g tomato fraction dry weight (µg/g dw).

8.2.9. Carotenoids in vitro *bioaccessibility*

The carotenoids *in vitro* bioaccessibility was measured the day after the processing following the procedure described by Minekus et al. (2014) with minor modifications. Five g of sample was weighed into a 50 mL capacity falcon tube. The sample was diluted with 4 mL of Simulated Salivary Fluid (SSF: 15.1 mL of 0.5 M KCl, 3.7 mL of 0.5 M KH_2PO_4, 6.8 mL of 1 M $NaHCO_3$, 0.5 mL of 0.15 M $MgCl_2(H_20)_6$, 0.06 mL of 0.5 M $(NH_4)_2CO_3$), 975 µL miliQ water, 7.24 mL of Simulated Gastric Fluid (SGF: 6.9 mL of 0.5 M KCl, 0.9 mL of 0.5 M KH_2PO_4, 12.5 mL of 1 M $NaHCO_3$, 11.8 mL of 2 M NaCl, 0.4 mL of 0.15 M $MgCl_2(H_20)_6$, 0.5 mL of 0.5 M of $(NH_4)_2CO_3$), 50 µL of 0.3 M $CaCl_2$ solution, 260 µL of freshly prepared L-α-phosphatidylcholine solution (10 mg/mL in SGF). The latter was obtained by preparing 50 mg/mL L-α-phosphatidylcholine (Sigma-Aldrich) solution in chloroform:methanol (1:1 v:v). To simulate the gastric digestion, the pH of the mixture was adjusted to 3 ± 0.05 with 1 M HCl or 1 M NaOH and 1.6 mL of porcine pepsin solution (25000 U/mL) was added. After flushing sample headspace with nitrogen for 10 s, the mixture was incubated at 37 °C for 2 hours while shaking end-over-end. Afterwards, to mimic the passage through the small intestine, 11

mL of Simulated Intestinal Fluid (SIF: 6.8 mL of 0.5 M KCl, 0.8 mL of 0.5 M KH_2PO_4, 42.5 mL of 1 M $NaHCO_3$, 9.6 mL of 2 M NaCl, 1.1 mL of 0.15 M $MgCl_2(H_20)_6$), 2.5 mL of fresh bile (0.089 g/ml in SIF), 40 µL of 0.3 M $CaCl_2$ and 5.0 mL of pancreatin (0.33 g/ml in SIF) were added. The pH of the solution was adjusted to 7 ± 0.05 with 1 M HCl or 1 M NaOH. Finally, the sample headspace was flushed with nitrogen for 10 seconds and the solution was incubated at 37 °C for 2 hours while shaking end-over-end. The digest was centrifuged (Beckman L-60 Ultracentrifuge, Palo Alto, CA, USA) at 162000 g for 67 min at 4 °C to separate the micelles containing the carotenoids. The supernatant was collected and carotenoids quantified according to the method described above. After the extraction procedure, the carotenoids extract was up-concentrated under vacuum using a rotary evaporator at 35 °C and re-dissolved in hexane:dichlorometane (4:1 v:v). The concentration factor was calculated by adding a specific amount of β-apo-8'-carotenale prior to evaporation. The carotenoid *in vitro* bioaccessibility (B/C) in each fraction and for each treatment was defined as the percentage ratio between the carotenoids concentration in the micelles (B) and the carotenoid concentration (C) of the corresponding fraction and treatment before digestion.

8.2.10. Total solids content

The total solids content was measured by gravimetric method (AOAC, 1995).

8.2.11. Data analysis

The results are the average of at least two measurements carried out on two replicated experiments ($n \geq 4$). Data are reported as mean value ± standard error. Statistical analysis was performed using R v.2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance, one way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p<0.05$).

8.3. Results and discussion

8.3.1. Effect of PEF on microstructure and conductivity of tomato fractions

Figure 8.2 shows the microstructure of untreated and PEF+HEAT treated tomato tissue, cells clusters, single cells and chromoplasts. In untreated tomato tissue, carotenoids were dispersed within the cells glued together through the middle lamella (Brett & Waldron, 1996; Moelants et al., 2014). The isolation procedure allowed to obtain in the cells clusters fraction a mixture of intact and broken cells and debris with carotenoids homogenously dispersed, while in single cells intact membranes can be observed. In chromoplasts fraction, a single thin membrane layer enveloped carotenoid crystals (Jeffery, Holzemburg, & King, 2012). PEF+HEAT caused cell detachment in tomato tissue, while in cells clusters and single cells, a large number of cells were damaged. In chromoplasts, no visual differences were found among untreated and PEF+HEAT treated samples. Moreover, no microstructural differences were found among the samples subjected to PEF+HEAT, PEF and HEAT (data not shown).

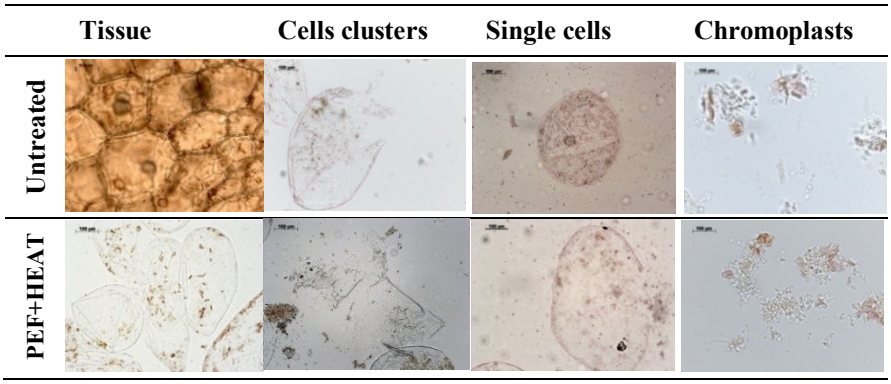


Figure 8.2. Micrographs of untreated and PEF+HEAT treated tissue, cells clusters, single cells and chromoplasts fractions obtained from tomatoes.

To understand the effect of PEF+HEAT, PEF and HEAT on the changes in cell membranes, conductivity of tomato fractions in a frequency range between 1 kHz and 1 MHz was measured. Figure 8.3 shows the conductivity spectra of untreated and PEF+HEAT treated tomato tissue as well as those relevant to samples subjected to PEF and HEAT treatments.

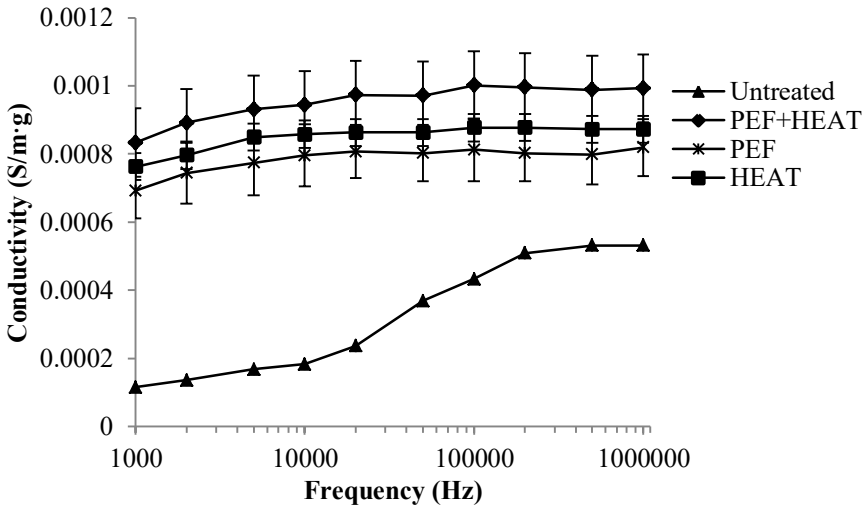


Figure 8.3. Conductivity (S/m·g) spectra of tomato tissue subjected to combined pulsed electric fields and heating (PEF+HEAT), pulsed electric fields (PEF) and heating (HEAT) only. Data relevant to untreated samples are also shown.

In untreated tomato tissue, low conductivity values were found at low frequency because the cell membrane acts as a capacitor, preventing the current electric flow into the medium. As expected, by increasing the frequency, the conductivity values increased due to the decreased resistance of the cell membrane upon the current flow applied during conductivity measurements. Upon PEF+HEAT, PEF and HEAT treatments, high

conductivity values were found in the whole frequency range, indicating a modification of the membrane permeability. In particular, the higher conductivity level in the low frequency indicated the irreversible membrane electroporation (Donsì et al., 2010). Under our process conditions, no significant differences ($p>0.05$) in conductivity were found among the treated samples. In order to obtain an indication of the proportion of permeabilized cells due to treatments, the disintegration index (Z_p) was calculated. The latter is the commonly accepted quantitative index of electroporation extent since it reflects the microstructural changes through the fractions (Angersbach, Heinz, & Knorr, 1999). In tomato tissue subjected (Angersbach, Heinz, & Knorr, 1999). Upon PEF+HEAT, Z_p reached the value of 0.8, indicating that most of the cells membrane were damaged. Similar Z_p values were found for PEF and HEAT (data not shown). Thus, although these results give an indication that cell membrane modification actually occurred as a consequence of processing, they do not allow to discriminate among the different cell damages. According to the literature, PEF treatments may cause pores formation in membrane, while thermal treatments induce cell wall pectin as well as cell membrane disruption (Moelants et al., 2014; Zimmermann, 1986).

In cells clusters, single cells and chromoplasts, the conductivity in the whole frequency range did not increase upon the treatments as compared to the untreated counterparts (data not shown). It is likely that samples preparation steps (e.g. blending and heating at 90 °C for 30 min) have already damaged cell membranes, causing an increase in conductivity to a saturation value. For this reason, no further conductivity increase was found for the treated tomato fractions, although membrane damage can be hypothesised as occurring under our process conditions. In fact, the application of electric field strength similar to that applied in this study showed modification of the cell permeability in plant tissue (Luengo et al., 2014; Janositz and Knorr, 2010; Wiktor et al., 2015).

8.3.2. Effect of PEF on carotenoids concentration and bioaccessibility

In order to understand the effect of PEF+HEAT treatment on bioactive compounds, carotenoids concentration and bioaccessibility were determined. Table 8.1 shows β-carotene and all-*trans* lycopene concentrations of untreated and treated tomato fractions.

Table 8.1. β-carotene and all-*trans* lycopene concentrations (µg/g dw) in tomato tissue, cells clusters, single cells and chromoplasts subjected to combined pulsed electric fields and heating (PEF+HEAT), pulsed electric fields (PEF) or heating (HEAT) only. Data relevant to untreated samples are also shown.

		Concentration (µg/g dw)	
Sample	**Treatment**	β-carotene	all-*trans* lycopene
Tissue	Untreated	32.2 ± 5.2 ^{a}	647.6 ± 65.9 $^{a'}$
	PEF+HEAT	40.6 ± 3.1 ^{a}	557.1 ± 58.1 $^{a'}$
	PEF	26.5 ± 2.3 ^{a}	463.8 ± 53.2 $^{a'}$
	HEAT	25.9 ± 2.2 ^{a}	588.2 ± 51.0 $^{a'}$
Cells clusters	Untreated	32.2 ± 1.3 ^{a}	346.8 ± 47.8 $^{a'}$
	PEF+HEAT	30.4 ± 0.9 ^{a}	345.5 ± 5.6 $^{a'}$
	PEF	16.9 ± 0.5 ^{c}	395.3 ± 2.8 $^{a'}$
	HEAT	24.2 ± 0.4 ^{b}	304.3 ± 1.0 $^{a'}$
Single cells	Untreated	75.7 ± 4.2 ^{a}	839.4 ± 119.4 $^{a'b'}$
	PEF+HEAT	68.4 ± 6.4 ^{ab}	825.9 ± 134.2 $^{a'b'}$
	PEF	52.6 ± 0.7 ^{bc}	1096.8 ± 31.7 $^{a'}$
	HEAT	45.5 ± 3.1 ^{c}	569.8 ± 69.1 $^{b'}$
Chromoplasts	Untreated	341.6 ± 5.9^{a}	2970.1 ± 44.0 $^{a'}$
	PEF+HEAT	187.3 ± 7.7 ^{c}	1881.7± 43.9 $^{b'}$
	PEF	255.8 ± 18.4 ^{b}	2191.7 ± 227.2 $^{b'}$
	HEAT	283.6 ± 1.3 ^{b}	2794.1 ± 46.3 $^{a'}$

$^{a, b}$: means with different letters within each tomato fraction indicate significant differences ($p<0.05$) for β-carotene

$^{a', b'}$: means with different letters within each tomato fraction indicate significant differences ($p<0.05$) for all-*trans* lycopene

It can be observed that the application of PEF+HEAT did not change β-carotene concentration in tomato tissue, cells clusters and isolated cells. However, a decrease of β-carotene concentration was found upon PEF or HEAT in cells clusters and single cells, likely due to oxidation and isomerization phenomena favoured by electrochemical reactions and metal release (Morren, Roodenburg, & de Haan, 2003; Pataro et al., 2014; Roodenburg et al., 2005). By contrast, no significant differences ($p>0.05$) in all-*trans* lycopene concentration were found in tissue, cells clusters and single cells upon the

application of the above technologies, probably due to the higher stability to oxidation and isomerization phenomena of lycopene compared to β-carotene (Lemmens et al., 2013; Seybold et al., 2004). These results are in agreement with the literature in the framework of PEF and heating. In particular, PEF treatment at 3-7 kV/cm did not affect the lycopene concentration in tomato tissue (Luengo et al., 2014). Similarly, temperature below 100 °C did not induce lycopene isomerization and degradation in tomato derivatives (Colle et al., 2010a; Nguyen & Schwartz, 1998; Sharma & Le Meguer, 1996). It is noteworthy that under our experimental conditions, temperature never exceeded 90 °C. In chromoplasts fraction, HEAT caused a decrease of about 21% in β-carotene concentration, while no changes were found in all-*trans* lycopene concentration. By contrast, both β-carotene and all-*trans* lycopene concentrations decreased by 47% and 36%, respectively, upon the application of PEF+HEAT or PEF. Such a decrease that is attributable to carotenoids oxidation and/or isomerization could be favoured by the higher susceptibility of carotenoids, enveloped in a thin membrane layer, towards oxygen permeability, metal release by the electrodes and electrochemical reaction occurring during PEF treatment (Jeffery et al., 2012; Morren et al., 2003; Pataro et al., 2014; Roodenburg et al., 2005). In order to understand the effect of PEF+HEAT, PEF and HEAT on the functional properties of the tomato fractions, carotenoids bioaccessibility was investigated. Carotenoids bioaccessibility indicates the carotenoids fraction released from the matrix and incorporated into micelles during the *in vitro* digestion. In our study, bioaccessibility was calculated as the ratio of the carotenoids incorporated into micelles after the digestion to the initial carotenoid concentration in the same processed sample. Figure 8.4 shows β-carotene and all-*trans* lycopene bioaccessibility in tomato fractions upon PEF+HEAT, PEF and HEAT. In untreated tissue, cells clusters and single cells the β-carotene and all-*trans* lycopene bioaccessibility ranged between 2% and 5%, in agreement with the literature (Palafox-Carlos et al., 2011; Palmero et al., 2013). As expected, lower β-carotene and all-*trans* lycopene bioaccessibility values were found in untreated tissue, cells clusters and single cells than in chromoplasts. It can be inferred that intact cell wall polysaccharides may prevent the digestive enzymes, bile salts and surfactants (i.e phospholipids) reaching the bioactive compounds within the chromoplasts and hampered micelle formation during intestinal step (Palafox-Carlos et al., 2011). Moreover, the higher carotenoids bioaccessibility in tissue compared to cells clusters and single cells could be attributed to the presence of chromoplasts leaked from the broken cells along the surface during the preparation step (i.e cutting). Moreover, from Figure 8.4A it can be also observed that in tissue β-carotene bioaccessibility did not change upon PEF+HEAT, PEF and HEAT. It is likely that, although the treatments caused the modification of cell permeability (Figure 8.1), the presence of multiple encapsulation barriers was enough to prevent the carotenoids release (Palafox-Carlos et al., 2011). All-*trans* lycopene bioaccessibility in tomato tissue were not affected by PEF, in agreement with Jayathunge et al. (2017), while a significant decrease was found in PEF+HEAT and HEAT treated samples. It can be inferred that heating induced the formation of a barrier consisting of cell wall and cell membrane that hinders the lycopene release (Palmero et al., 2014). Differences in β-carotene and all-*trans* lycopene bioaccessibility upon the treatments can be attributable to their different molecular

structure. In fact, the linear isoprenoid chain confers to lycopene lower solubility capacity compared to β-carotene and thus lower bioaccessibility (Tyssandier, Lyan, & Borel, 2001; Van het Hof et al., 2000; Gupta et al., 2011; Sánchez-Moreno et al., 2004).

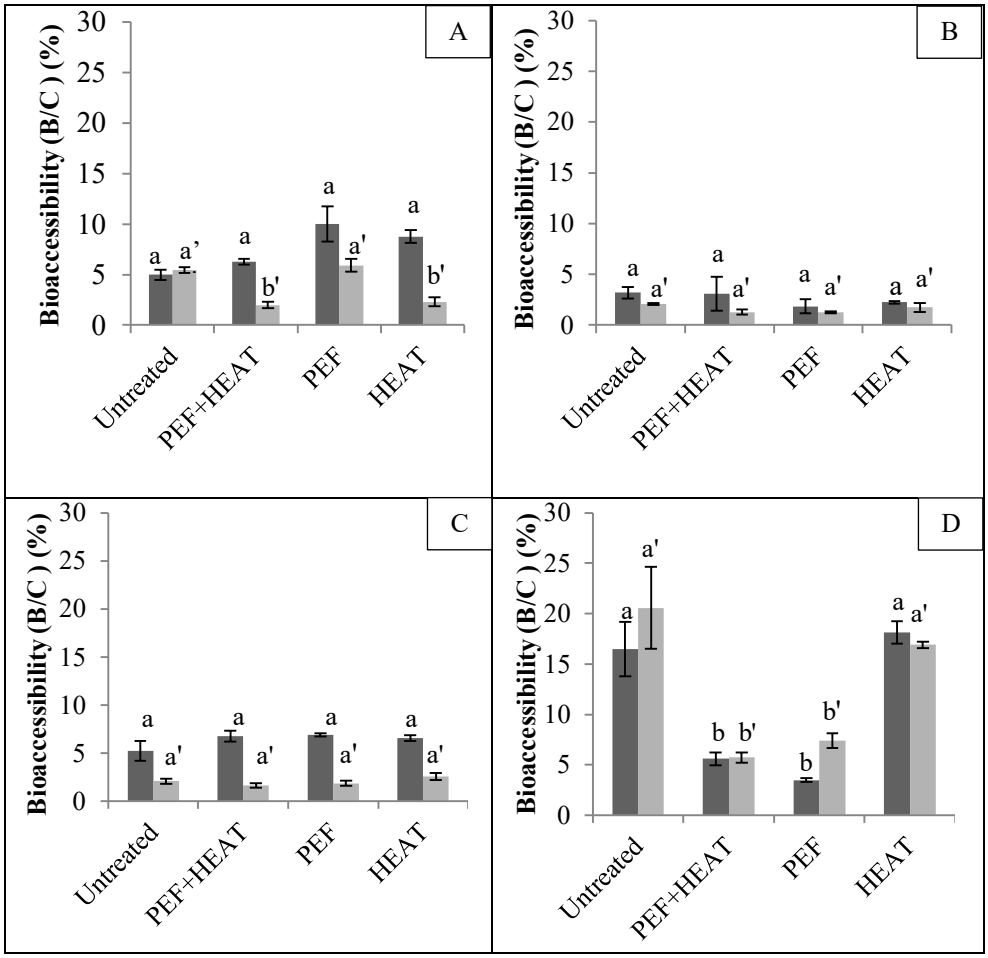


Figure 8.4. β-carotene ( ) and all-*trans* lycopene ( ) bioaccessibility (B/C %) in tissue (A), cells clusters (B), single cells (C) and chromoplasts (D) subjected to combined pulsed electric fields and heating (PEF+HEAT), pulsed electric fields (PEF) or heating (HEAT) only. Data relevant to untreated samples are also shown.

[a, b,] Means with different letters within each tomato fraction indicate significant differences ($p<0.05$) for β-carotene

[a', b'] Means with different letters within each tomato fraction indicate significant differences ($p<0.05$) for all-*trans* lycopene

In cells clusters and single cells (Figures 8.4B and 8.4C), β-carotene and all-*trans* lycopene bioaccessibility did not change upon PEF+HEAT, PEF and HEAT. It is likely that cellular barriers present in the sample (i.e cells clusters) or induced by thermal

treatments during the sample preparation (i.e. single cells) did not allow to modify the carotenoids release and incorporation into biliary micelles during in vitro digestion (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011; Palmero et al., 2014). To understand the role of membranes, carotenoids bioaccessibility was studied in the chromoplasts fraction (Figure 8.4D), which had a single membrane layer. In untreated chromoplasts fraction, β-carotene and all-*trans* lycopene bioaccessibility ranged between 16% and 20%. The application of PEF+HEAT caused a significant reduction of carotenoids bioaccessibility to 5%. Similar results were obtained for the PEF treated chromoplasts. On the contrary, HEAT did not cause significant modifications ($p>0.05$) of carotenoids *in vitro* bioaccessibility, in agreement with Colle et al. (2010a).
The reduction in carotenoids bioaccessibility at chromoplasts level can be attributable to the effect of PEF rather than to HEAT and might suggest that PEF induced modifications not only in the membrane but also in the carotenoids-protein complexes. In fact, it is well known that carotenoids are tightly bound to subcellular lipids and binding proteins within the chromoplast structure (Faulks & Southon, 2005) and that complexes constitute a further structural barrier for carotenoids bioaccessibility. Moreover, Perez and Pilosof (2004) found that PEF treatment can induce modification in protein conformation. Although further research is needed, these results clearly show a role of PEF on carotenoids bioaccessibility.

8.4 Conclusions

The results of this study show that PEF treatment applied individually or in combination with heating induced changes of the microstructure and membrane permeability of tomato fractions. These treatments caused slight or no changes in β-carotene concentration and bioaccessibility in tissue. Similarly, all-*trans* lycopene concentration and bioaccessibility did not change upon PEF. A decrease of all-*trans* lycopene bioaccessibility upon PEF+HEAT and HEAT was found, suggesting the formation of a barrier that hinders carotenoids release. In cells clusters and single cells, slight or no changes were found in carotenoids concentration and bioaccessibility. On the contrary, in chromoplasts both β-carotene and all-*trans* lycopene concentration and bioaccessibility significantly decreased upon PEF with or without heating due to PEF related phenomena. This effect was attributed to the comparatively higher susceptibility of the chromoplasts fraction towards PEF treatment compared to tomato fractions which possess further structural barriers. It can be concluded that the effect of PEF on carotenoids bioaccessibility strongly depends on the vegetable structure complexity and by structural barriers either naturally present or induced by the process that impede the carotenoids release. The results of this study clearly indicate individual PEF can be applied to tomato based products, mainly constituted by tissue, cells clusters and single cells, without impairing carotenoids functionality.

Chapter 9

General discussion

9.1. Introduction

Nowadays, food processing is aimed to provide food products which are not only safe and sensory acceptable, but also to steer food quality and nutritional functions. Therefore, in this context, the challenge for food scientists is the proper selection of processing and process parameters.

Over the last decades, unconventional technologies have gained more and more interest in the food sector. Taking advantages of driven forces (mechanical, electromagnetical, chemical) different from thermal treatment, unconventional technologies can be used for the production of foods with enhanced quality and specific nutritional functions. Although several studies have been already published on the effect of unconventional technologies on foods, to better understand the process mechanism and find the process conditions leading to the desired effects, further research is needed.

In this Ph.D. thesis, US, HPH and PEF were selected as unconventional technologies and their effect on quality (Part I) and functionality (Part II) in fruit and vegetable derivatives was investigated. In particular, the first part describes the effect of HPH, US and their combination on polyphenoloxidase activity in apple juice, physical properties of tomato juice, microbial decontamination in wastewater deriving from fresh-cut vegetables. The second part was addressed to study the effect of HPH, US and PEF on *in vitro* functionality. HPH was applied for the production of an oil-in-water nanoemulsion delivering silybin and the effect of oil type on silybin *in vitro* bioaccessibility was investigated. Moreover, the effect of US on functional properties (α-glucosidase inhibitory activity and lycopene bioaccessibility) of tomato juice was studied. Finally, the effect of PEF on carotenoids bioaccessibility in tomato fractions characterized by different physical barriers was investigated.

9.2. Main findings

In Chapter 2, the results acquired showed that HPH treatments applied individually or in combination with US were scarcely efficient in inactivating PPO in apple juice. On the other hand, low frequency US treatments applied with or without temperature control resulted more effective in inactivating PPO than HPH treatments. In particular, the complete PPO inactivation was achieved upon US without temperature control. Under these processing conditions, *in situ* heat generated during US treatment greatly contributed to enzyme inactivation.

In Chapter 3, the results evidenced that HPH and US treatments were responsible for higher G' and consistency of processed tomato juices than untreated samples. Increases in sample rheological properties were comparable for the 5.0 and 7.5 °Brix treated HPH and US samples, whereas HPH was more effective than US in the case of 10.0 °Brix sample. These changes were in agreement with other indexes (precipitate weight ratio increase and cell integrity decrease) and were attributed to stronger inter-particle

interactions. Moreover, the process energy efficiency showed that lower energy was involved in HPH in comparison to US.
In Chapter 4, the results showed that the application of US could represent an effective tool for wastewater decontamination in the fresh-cut industry. In particular, US supplied without temperature control allowed to achieve 3.2 Log reductions of native microflora during 20 min treatment, while 5 Log reductions of inoculated *L. monocytogenes*, *E. coli* and *S. enterica* were attained within 5 min of ultrasonication.
In Chapter 5, the results acquired showed that HPH treatment allowed to obtain stable nanoemulsion enriched with silymarin. During storage, silybin, the main bioactive compounds in silymarin extract, underwent degradation, showing lower stability in extra virgin oil and sunflower oil than in castor oil. On the other hand, the oil type slightly influenced the silybin *in vitro* bioaccessibility.
In Chapter 6, high frequency US did not affect lycopene and polyphenol concentration as well as the functional properties of tomato juice.
In Chapter 7, low frequency US treatment caused an increase in viscosity in tomato juice; however these changes did not affect lycopene bioaccessibility. Moreover, lycopene bioaccessibility significantly decreased with the increase of oil content in both untreated and ultrasonically treated tomato juice. No differences in lycopene *in vitro* bioaccessibility were found between the untreated and US treated samples. Losses of lycopene *in vitro* bioaccessibility occurred in the untreated and ultrasonically treated tomato juice with and without oil during storage, mainly due to carotenoid oxidation.
In Chapter 8, PEF treatments applied individually or in combination with heating caused modification of cells membrane permeability. Carotenoids concentration and bioaccessibility was slightly affected by PEF treatment in tomato cells, cells clusters and single cells. On the other hand, a significant decrease in carotenoids concentration and bioacessibility was found in the chromoplasts fraction, probably due to the higher susceptibility of carotenoids in the chromoplasts compared to tomato fractions with increasing number of physical barriers (i.e. tissue, cells clusters and single cells).

9.3. Methodological considerations and recommendations

9.3.1. Unconventional technologies

The outcomes of this research highlighted that unconventional technologies, namely HPH and US, caused PPO inactivation, physical properties modification and microbial decontamination. However, it is evident that to obtain the desired quality changes, the selection of proper process and process conditions is crucial. To this regard, some considerations about the application of unconventional technologies and process parameters selection can be highlighted. Several studies reported the effect of unconventional technologies on food quality, however, contrasting information can be found in the literature. In addition, the comparison among the results is difficult due to poor description of operating protocols. To tackle these issues, Raso et al. (2016) provided recommendations for standardization of research methods as well as key information that should be reported in studies regarding the application of PEF. According to the Authors, to fully describe PEF treatment, process parameters (i.e.

electric field strength, treatments time, pulse duration, frequency, energy density, total specific energy input, temperature), treatment chamber and medium characteristics have to be reported.

To our knowledge, similar guidelines for HPH and US treatments are not available. However, it is noteworthy that the key information to be reported in the operating protocols for HPH treatments are valve geometry and flow rate, while frequency, amplitude, vessel geometry, horn diameter are the main process parameters for US treatment. Next to these process parameters, during US treatment, temperature may increase of several degrees and thus significantly contributes to food quality changes. Therefore, in order to distinguish the mechanical contribution from the thermal one, a reference thermal treatment following the same time-temperature profile of the unconventional treatment has to be reported.

The results acquired in this thesis evidenced that it is possible to discriminate the acoustic and thermal contribution during US treatment. In particular, under controlled temperature regime the acoustic contribution to PPO inactivation was estimated for 18%, while the thermal one was accounted for approximately 73%. Similarly, the acoustic and thermal contributions to *L. monocytogenes* inactivation were estimated to account for about 22% and 58%, respectively. On the other hand, under uncontrolled temperature regime, PPO inactivation and microbial decontamination were mainly attributed to the thermal effect, while the acoustic effect was negligible.

Moreover, in this Ph.D. thesis, the effects of HPH and US treatments on PPO inactivation and modification of physical properties were also compared. US allowed a greater PPO inactivation compared to HPH, while HPH resulted more efficient than US in modifying physical properties of tomato juice. Differences in the observed effects between samples subjected to HPH and US can be attributed to different mechanical stresses generated by the two technologies.

The application of combined unconventional technologies was also considered as strategy for increasing their effect and, at the same time, reducing the energy input. In this context, more complex considerations arose with respect of the use of combined technologies due to difficulties in the proper selection of process parameters. Our findings evidenced that combined HPH and US treatments did not induce further PPO inactivation in apple juice compared to individual HPH and US treatment. On the other hand, the outcomes of this research highlighted that US combined with heating resulted an effective tool for both enzyme inactivation and microbial decontamination due to a synergistic effect between US and heating.

9.3.2. In vitro *functionality*

At the beginning of this research activity, data on the effect of unconventional technologies on *in vitro* functionality were scarce. For this reason, the second part of this thesis was addressed to study the effect of HPH, US and PEF on selected functional properties of fruit and vegetable derivatives. Together with the effect of unconventional technologies, the influence of oil type and amount, and storage conditions were investigated.

The results acquired evidenced that HPH treatment represents an effective technological tool for the development of functional foods such as oil-in water nanoemulsion containing silybin. In particular, silybin bioaccessibility was approximately 25-30% and the different type of oil did not affect sylibin *in vitro* functionality. However, although nanoemulsions represent a promising system for delivering bioactive compounds, it would be interesting to investigate silybin bioaccessibility when incorporated in more complex foods such as beverages, salad dressing and powder.

Regarding the effect of US and PEF on food functionality, our findings evidenced that although these technologies caused modification of physical properties of tomato cells (i.e. cells disruption or cell membranes permeabilization), the presence of multiple physical barriers is enough to prevent the carotenoids release and their incorporation into biliary micelles during *in vitro* digestion.

Moreover, the effect of PEF on chromoplasts, the structure constituted by a thin membrane layer where carotenoids are stored, were also investigated. Under our experimental conditions, a decrease in carotenoids bioaccessibility were found upon PEF treatment. This decrement might suggest that carotenoids became entrapped by process induced barriers, thus hindering their subsequent incorporation into micelles during the digestion. However, further research is needed to elucidate the factor that induce a decrease on carotenoids bioaccessibility at chromoplast level upon PEF treatment.

Alongside the effect of unconventional technologies on food functionality, the amount of oil added and storage play an important role in carotenoid functionality in tomato juice. The presence of oil allowed an increase of carotenoids bioaccessibility due to their lipophilic characteristics, however, high level of lipids can induce a decrease in carotenoids bioaccessibility due to the limited lipolysis during the *in vitro* digestion. Moreover, storage negatively affected lycopene bioaccessibility due to carotenoids degradation.

Therefore, the results acquired evidenced that unconventional technologies can steer food quality without affecting the functional properties. However, in order to meet the consumers' demand for food with improved nutritional functions, amount of oil and storage conditions have to be taken into account.

9.4. Future directions

To clarify the effect of unconventional technologies on fruit and vegetable derivatives and thus favour their industrial application, some of the future directions of research could include the following points.

- Scaling-up of the process. Since most of the studies are carried out on lab-scale equipment and the effectiveness of the process depends on the scale, the scaling-up of the process results essential for the industrial application of unconventional technologies. To our knowledge only industrial PEF systems are available, while further research is needed for industrial application of US and HPH.
- Determination of energy consumption and environmental impact. Available data on energy consumption, gas emissions, wastewater production, environmental sustainability of unconventional technologies are still scarce.
- Validation of *in vitro* studies with *in vivo* studies. *In vitro* studies are cheap, rapid and circumvent ethical issues, however *in vivo* tests are crucial for a better understanding of the potential benefits of unconventional technologies on humans health.

9.5. Main conclusions

This thesis described the effect of unconventional technologies on selected food quality and functional properties of fruit and vegetable derivatives. Starting from the effect of HPH, US and their combination on PPO inactivation in apple juice, the effect of HPH or US on physical properties of tomato juice and the effect of US on microbial inactivation in wastewater from fresh-cut vegetables, passing through the application of HPH for designing stable nanoemulsion delivering silybin, ending with the effect of US on polyphenol content and α-glucosidase inhibitory activity, lycopene concentration and *in vitro* bioaccessibility in tomato juice and finally the effect of PEF on carotenoids *in vitro* bioaccessibility in tomato fractions.

The results highlight that unconventional technologies are interesting strategies for steering food quality and functionality. However, the fundamental understanding of the process and the selection of process parameters are crucial for their future application at industrial level.

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List of additional publications

Papers

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