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Fruit beverages processed using ultrasound technology: physical properties, stability and enzyme inactivation

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Dissertation presented to obtain the degree of Master in Science. Area: Food Science and Technology

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RESUMO

Bebidas de frutas processadas utilizando tecnologia de ultrassom: Propriedades físicas, estabilidade e inativação enzimática

Neste trabalho estudou-se a melhoria na estabilidade, propriedades físicas e inativação enzimática em bebidas de frutas através da aplicação da tecnologia de ultrassom (US). Na primeira parte, foi avaliado o efeito do US no processamento de suco de pêssego. As alterações macroscópicas na estabilidade de sedimentação da polpa, turbidez, cor e propriedades reológicas foram analisadas. Foi demonstrado que a melhoria em cada uma das propriedades evidenciadas macroscopicamente envolve interação de mecanismos complexos que dependem diretamente de alterações microscópicas, tais como estrutura, tamanho, composição e interação entre as fases contínua (soro) e dispersa (polpa) do suco. Estas alterações foram avaliadas por microscopia e análise de distribuição de tamanho de partículas. Na segunda e terceira partes, a inativação da enzima peroxidase (POD) foi avaliada em água de coco. O efeito da aplicação do US na POD de água de coco foi estudado pela primeira vez, utilizando dois tipos de equipamentos (banho e sonda de US). Demonstrou-se que as alterações na atividade enzimática durante o processamento com US estão relacionadas às diferentes conformações que a enzima pode adotar, dependendo principalmente da energia aplicada ao sistema. Na terceira parte, o ultrassom foi então aplicado como pré-tratamento ao processamento térmico. A avaliação foi realizada sob condições não isotérmicas, sendo a cinética de inativação da POD modelada usando a função de distribuição de Weibull. Foi observado que o pré-tratamento com US diminuiu a atividade enzimática. Além disso, o efeito do US resultou em uma população de enzimas mais homogênea e termosensível, reduzindo significativamente o tempo necessário para o processamento térmico. Desta forma, este trabalho estudou e demonstrou que a tecnologia de ultrassom é uma alternativa interessante para melhorar as propriedades físicas e a estabilidade enzimática de bebidas à base de frutas, indicando sua importância tanto acadêmica quanto industrial.

Palavras-chave: Engenharia de alimentos; Tecnologia de ultrassom; Processamento térmico; Bebidas de frutas, Propriedades físicas, Inativação enzimática

ABSTRACT

Fruit beverages processed using ultrasound technology: physical properties, stability and enzyme inactivation

This work studied the improvement of stability, physical properties and enzymatic inactivation in fruit beverages by applying the ultrasound technology (US). In the first part, the effect of the US application on peach juice processing was evaluated. The macroscopic changes on pulp sedimentation stability, turbidity, colour and rheological properties were evaluated. As a result, it was demonstrated that the improvement in each of the properties evidenced at the macroscopic level involves interaction of complex mechanisms which depend directly on changes at the microscopic level, such as the structure, size, composition and interaction between the continuous phase (serum) and dispersed phase (pulp) of the juice. These changes were assessed by microscopy and particle size distribution. In the second and third parts, the inactivation of the enzyme peroxidase (POD) was evaluated in coconut water. In the second part, the effect of the application of US was evaluated for first time for coconut water POD, using two types of ultrasonic equipment (US baht and US probe). It was demonstrated that the changes of enzyme activity during the US process depend on the many forms that the enzyme can adopt, mainly depending on the energy applied to the system. Subsequently, in the third part, the US was applied as a pre-treatment of subsequent thermal processing. The evaluation was carried out under non isothermal conditions, being the POD inactivation kinetics modelled using the Weibull distribution function. Finally, it was observed that the pre-treatment using ultrasound slightly decreased the enzyme activity. Furthermore, the ultrasound effects resulted in a more homogeneous population and heat-sensitive enzymes, significantly reducing the needed time of thermal processing. In conclusion, this work studied and demonstrated that the ultrasound technology is an interesting alternative to improve the physical properties and enzymatic stability in fruit beverages, reflecting the importance from both the academic and industrial point of view.

Keywords: Food engineering; Ultrasound technology; Thermal processing; Fruit beverages; Physical properties; Enzyme inactivation

RESUMEN

Bebidas de frutas procesadas utilizando tecnología de ultrasonido: propiedades físicas, estabilidad e inactivación enzimática

En el presente trabajo se estudió la mejora de la estabilidad, propiedades físicas e inactivación enzimática en bebidas de frutas mediante la aplicación de la tecnología de ultrasonido (US). En la primera parte de esta investigación, se evaluó el efecto del procesamiento con US en jugo de durazno. Las alteraciones macroscópicas en la estabilidad de sedimentación de pulpa, turbidez, color y propiedades reológicas; fueron evaluadas. Como resultado se obtuvo que la mejora en cada una de las propiedades a nivel macroscópico implica interacción de mecanismos complejos que a su vez dependen directamente de los cambios a nivel microscópico en la estructura, el tamaño, composición e interacción entre la fase continua (suero) y la fase dispersa (pulpa) del jugo. Estos cambios fueron analizados mediante microscopia óptica y distribución de tamaño de partícula. En la segunda y tercera parte, se estudió la inactivación de la enzima peroxidasa (POD) en agua de coco, en donde se evaluó por primera vez el efecto de la aplicación del US. Primero se evaluó el efecto del US en la modificación de la actividad enzimática, utilizando dos tipos de equipos (baño y sonda de US). En esta parte se demostró que los cambios en la actividad enzimática durante el proceso con US depende de la distinta conformación que la enzima puede adoptar dependiendo de la energía ultrasónica aplicada en el sistema. Posteriormente, en la tercera parte, el US fue aplicado como pre tratamiento al procesamiento térmico. La evaluación se llevó a cabo en condiciones no isotérmicas y la cinética de inactivación de la POD fue modelada utilizando la función de distribución de Weibull. Finalmente, se observó que el pre procesamiento con ultrasonido disminuye la actividad enzimática. Adicionalmente, el efecto del US resultó en una población de enzimas más homogénea y sensible al calor, reduciendo significativamente el tiempo necesario para el procesamiento térmico. En conclusión, en esta investigación se estudió y demostró que la tecnología de ultrasonido es una buena alternativa para mejorar las propiedades física y estabilidad enzimática en bebidas, reflejando importancia desde el punto de vista académico e industrial.

Palabras clave: Ingeniería de alimentos; Tecnología de ultrasonido; Proceso térmico; Bebidas de frutas; Propiedades físicas; Inactivación enzimática

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LIST OF ACRONYMS AND ABBREVIATIONS

AA = Ascorbic acid (Table 1.3 and 1.4)

APx = Ascorbate peroxidase

DPCD = Dense Phase Carbon Dioxide (Table 1.2)

HHP = High Hydrostatic Pressure (Table 1.2 and 1.4)

HP = High Pressure (Table 1.2)

HPCD = High Pressure Carbon Dioxide (Table 1.2)

HPH = High Pressure Homogenization

MF = Microfiltration (Table 1.3)

MS = Mano-sonication

MTS = Mano-thermo-sonication

MW = Microwave heating (Table 1.2 and 1.3)

PEF = Pulsed Electric Fields (Table 1.2)

PME = Pectinmethylesterase

POD = Peroxidase

PPO = Polyphenol oxidase

PS = Photo-sonication

PSD = Particle Size Distribution

PTS = photo-thermo-sonication (Table 1.4)

SCD = Supercritical Carbon Dioxide (Table 1.2)

TS = Thermo-sonication (Table 1.4)

TT = Thermal process (Table 1.2, 1.3 and 1.4)

UA = combined process with US and AA (table 1.4)

UF = Ultrafiltration (Table 1.3)

UHT = Ultra-high temperature

US = Ultrasound

UV = Ultraviolet irradiation (Table 1.2 and 1.3)

SYMBOLS LIST

CHAPTER 1

$\propto$ = relative enzyme resistance (eq. (1.6)) [-]

A_0 = initial enzyme activity for $t = 0$ (eq. (1.5)) [-]

A_∞ = residual activity after prolonged treatment time (eq. (1.7)) [-]

A_t = enzyme activity during the time processing (eq. (1.5)) [-]

$\frac{A_t}{A_0}$ = residual enzyme activity (eq. (1.5)) [-]

Cp = specific heat capacity (eq. (1.2)) [J/g.°C]

D_T = decimal reduction time value (eq. (1.11), (1.14)) [s or min]

D_{Tref} = decimal reduction time at T_{ref} (eq. (1.14)) [s or min]

f = wave frequency (eq. (1.1) [kHz]

$F(t)$ = time of failure according Weibull distribution (eq. (1.9))

k, k_s, k_R, k_1, k_2 = kinetic parameters (eq. (1.5), (1.6), (1.7), (1.8), (1.12)) [1/s]

Λ = ratio between the activities of the native and intermediate states (eq. (1.8)) [-]

λ = wavelength (eq. (1.1)) [nm]

m = mass (eq. (1.2)) [g]

P = ultrasound power (eq. (1.2)) [W]

p, b = scale parameters of Weibull distribution (eq. (1.9), (1.10)) [Different units]

q, n = shape parameters of Weibull distribution (eq. (1.9), (1.10)) [Different units]

r = probe tip with radius (eq. (1.3)) (cm^2)

SSE = sum of squared errors (eq. (1.15))

S_{wave} = speed of the wave (eq. (1.1)) [m/s]

t = time [min]

T = temperature [°C]

t_{equiv} = equivalent holding time (eq. (1.13, (1.14)) [min or s]

T_{ref} = temperature of reference (eq. (1.13), and (1.14)) [°C]

$T_{(t)}$ = time-temperature history (eq. (1.13), (1.14))

$U_{density}$ = ultrasound power density (eq. (1.4)) [W/cm^3]

$U_{intensity}$ = ultrasound power intensity (eq. (1.3)) [W/m^2]

V = volume [cm^3 or mL]

z = temperature increase that reduces D_T by 90% (eq. (1.14)) [°C]

CHAPTER 2

$a*$ = axis of chromaticity between green (-) and red (+) (eq. (2.4))

$b*$= axis between blue (-) and yellow (+) (eq. (2.4))

$D[4,3]$ = volume-based mean diameter (eq. (2.1)) [μm]

$D[3,2]$ = area-based mean diameter (eq. (2.2)) [μm]

d_i = particle diameter (eq. (2.1) and (2.2)) [μm]

ΔE = total colour difference (eq. (2.4))

IS = sedimentation index (eq. (2.3)) [%]

$IS_{initial}$= initial sedimentation index [%]

$IS_{equilibrium}$= sedimentation index in the equilibrium [%]

k = consistency index in the Herschel-Bulkley model (eq. (2.6)) [Pa·s^n]

k = kinetic parameter of pulp sedimentation model [1/days]

k_{FS} = kinetic parameter in the Figoni-Shoemaker model (eq. (2.6)) [1/s]

k_B = Boltzmann constant (eq. (2.8)) [= 1.38·10^{-23} N·m·K^{-1}]

$L*$ = lightness parameter ($L*$= 0 or 100 indicates black or white) (eq. (2.4))

$\eta_{continuous\_phase}$ = viscosity of the serum phase (eq. (2.8)) [Pa·s]

η = viscosity [Pa·s]

η_a = apparent viscosity ($= \sigma/\dot{\gamma}$) [Pa·s]

n_i= number of particles (eq. (2.1) and (2.2))

n = flow behaviour index in the Herschel-Bulkley model (eq. (2.5)) [-]

σ_e = equilibrium stress in the Figoni-Shoemaker model (eq. (2.5)) [Pa]

σ_i = initial stress in the Figoni-Shoemaker model (eq. (2.5)) [Pa]

σ_0= yield stress in the Herschel-Bulkley model (eq. (2.6)) [Pa]

σ = shear stress [Pa]

Pe = Peclet number (eq. (2.8)) [-]

$\bar{r}_{particle}$= means suspended particle radius (eq. (2.8)) [m]

$\dot{\gamma}$= shear rate [s^{-1}]

$S_{(t)}$= sediment volume measured along the storage time (eq. (2.3)) [mL]

t = storage time (eq. (2.3)) [days]

t = time (eq. (2.5)) [s]

T = absolute temperature (eq. (2.8)) [K]

V = total volume of the sample (eq. (2.3)) [mL]

CHAPTER 3

A = enzyme activity (eq. (3.2))

Abs_0 = initial absorbance (eq. (3.1) and (3.2))

Abs_∞ = maximum constant absorbance (eq. (3.1) and (3.2))

$Abs_{(t_{Abs})}$ = absorbance at 420 nm (eq. (3.1))

$\frac{A_{(tUS)}}{A_0}$ = relative or residual activity during ultrasound processing time

E_0 = initial enzymatic state (eq. (3.3))

E_n = n intermediate state of the enzyme after application of energy ($\varepsilon_{(n-1)-(n)}$) (eq. (3.3))

$\varepsilon_{(n-1)-(n)}$ = energy applied to pass of $E_{(n-1)}$ to E_n enzymatic state (eq. (3.3))

k_{Abs} = kinetic parameter (eq. (3.1) and (3.2)) [1/s]

P = ultrasonic power [W]

t_{US} = is the ultrasound processing time [s].

t_{Abs} = time measurement of absorbance readings (eq. (3.1)) [s]

U_{ec} = ultrasonic energy consumption [J/mL]

CHAPTER 4

A_0 = initial enzyme activity

A_0' = enzymatic activity after 30 min of sonication (eq. (4.8)) [-]

A_t = enzyme activity during thermal processing

A_{tUS} = enzyme activity during ultrasound processing

β = parameter that indicates the shape of the inactivation curve (eq. (4.3)) [-]

δ_T = time for the first decimal reduction at a given temperature T (eq. (4.3)) [s]

δ_{Tr}= time for the first decimal reduction at reference temperature T_r (eq. (4.4)) [s]

F_{T_r} = equivalent processing time at T_r (eq. (4.6), (4.7), (4.9)) [s]

λ = enzymatic activity reduction after sonication ($= A_0'/A_0$) (eq. (4.7)) [-]

$L_t(t)$ = lethality function (eq. (4.5)) [-]

t = process time [min or s]

T_r = reference temperature (eq. (4.4), (4.5), (4.6)) [°C]

$T(t)$ = temperature history (eq. (4.5), (4.6))

TT = Thermal processing

US-TT = US application as a pre-treatment to subsequent TT

z = temperature change required to cause a tenfold change in δ_T and δ_{Tr} (eq. (4.4), (4.5), (4.6)) [°C]

1 INTRODUCTION*

Ultrasound is considered an emerging technology that can be used as alternative to conventional food processing with a wide range of application. Low power ultrasound can be used in diagnosis applications to characterize food medium. When the applied ultrasound power is high, it arises interesting physical, and chemical effects, which are capable of altering the material properties (e.g., disrupting the physical integrity, acceleration of certain chemical reactions) through generation of pressure, shear, and temperature gradients in the medium through which they propagate. Based on that, the aim of this study is the application of high power ultrasound on food, focusing this technology on the stability, physical properties and enzymatic inactivation in fruit beverages.

The main physical properties of fruit juices are affected by changes on its composition and structure at the molecular and microscopic levels, and are associated with the propensity to separate into layers, appearance of sediments, flocs or particulates, cloudiness, colour, texture, rheological characteristics, etc. The physical properties and stability are good indicators of quality where undesirable changes traditionally avoided by adding stabilizers, reduce the consumer acceptance. On the other hand, the enzymatic activity, such as of peroxidase (POD), is detrimental to the quality of processed products of fruits and vegetables resulting in effects such as browning, loss of flavour and vitamins. In this study, the coconut water was selected to study the enzymatic inactivation. It can be considered as a special product, where despite different methods and processing technologies were applied, the high enzyme stability is still a challenge for the product industrialization. In coconut water, the enzymes naturally present showed a higher resistance, and a no severe thermal process may not be sufficient for enzymatic inactivation due to the enzyme thermal resistance. Also high temperatures and processing times can cause loss of nutrients and induce the formation of compounds with a roasted and malted flavour, while filtration techniques reduce different nutrients of fresh coconut water.

In the present section of introduction, is presented the literature review that contains the basic concepts of the ultrasound (US) technology, physical properties, stability and enzyme activity, for a better global understanding of this work. Next, the

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* Part of this chapter composes the following book chapter, which is currently in press as: **Ultrasonic processing of fruit and vegetable juices**. In: *Ultrasound: Advances for Food Processing and Preservation.*

results are presented in three chapters, which are divided in two parts for better organization (Figure 1.1).

Part 1 Physical Properties		Part 2 Enzyme Inactivation		
CHAPTER 1. Introduction	**CHAPTER 2.** Ultrasound technology to improve the physical properties and stability of fruit juices	**CHAPTER 3.** The ultrasound technology for modifying enzyme activity	**CHAPTER 4.** Using the ultrasound technology for the inactivation and thermal sensitization of peroxidase (POD) in coconut water	**CHAPTER 5.** General conclusions

Figure 1.1 – General structure of the dissertation development

The first part consists on the ultrasound technology application as a possible processing to improve the peach juice stability and physical properties (presented in the chapter 2). Where was evaluated the changes produced by ultrasound technology on juice microstructure, rheological properties of juice and serum, sedimentation stability, turbidity and colour during storage and mathematical modelling of relevant evaluated properties.

In the second part, the ultrasound technology was applied in green coconut water and was evaluated the POD inactivation. Where was firstly evaluated and studied the influence of two different ultrasonic equipment (ultrasound probe and bath), in the enzyme activity behaviour (presented in chapter 3). And in the chapter 4, was evaluated the POD sensitization with US pre-treatment to improve the conventional thermal processing. Finally, is presented the general conclusions, as well as suggestions for future studies.

1.1 Ultrasound technology

The ultrasound (US) technology is considered an emerging technology, with an environmental claim, and has been proposed as an alternative to the conventional food processing. It is based on the propagation of mechanical waves throughout the product. The ultrasonic range is considered to be at frequencies over 20 kHz. In contrast, sound waves with frequency in the range of 16 Hz to 18 kHz are in the audible

range and sound waves with frequency below 16 Hz are in the infrasonic range (RASTOGI, 2011). The ultrasonic waves are characterized by their frequency (f) and wavelength (λ), being the product of these the speed of the wave (S_{wave}) (eq. 1.1). Acoustic waves are mechanical waves that need a material medium to propagate, by the motion of the disturbance through the medium. These mechanical waves can propagate in solids, liquids, and gases (MICHAEL; LU; KATHRYN, 2005).

$$S_{wave} = f . \lambda \qquad (1.1)$$

The ultrasound generation is carried out through transducers. There are three types of ultrasonic transducers in common usage including liquid-driven transducers, magnetostrictive transducers, and piezoelectric transducers (most common devices used for the ultrasound generation, and contain ceramic and piezoelectric materials such as barium titanate or lead metaniobate). There are numerous types of ultrasonic equipment available, which can be used as sonochemical reactors. These include ultrasonic bath, ultrasonic probe, parallel or radial vibrating system, etc. Basically, the ultrasonic bath is a tank that contains a process medium with transducers bonded to its base. For ultrasonic baths, power is often low in order to avoid cavitational damage to the tank walls and the power density is low due to a large volume of the processing liquid. On the other hand, the ultrasonic probe system are used to amplify the acoustic energy generated from a transducer (MASON, 1998). For the classification of ultrasound applications, the energy amount of the generated sound field is the most important criterion. It is characterised by sound power (W), sound intensity (W/m^2) or sound energy density (W/cm^3 – also called the volumetric power) (KNORR et al., 2004). Ultrasonic intensity or acoustic energy density can be determined calorimetrically using the following equations (O'DONNELL et al., 2010; FONTELES et al., 2012).

The absolute ultrasonic power (P) is given as:

$$P = mCp \left(\frac{dT}{dt} \right) \qquad (1.2)$$

Where m is the sample mass, Cp is the specific heat capacity, and $(\frac{dT}{dt})$ is the rate of change of temperature during sonication.

The intensity of ultrasonic power ($U_{intensity}$) dissipated from a probe tip with radius (r) is given by:

$$U_{intensity} = \frac{P}{\pi.r^2} \qquad (1.3)$$

The acoustic energy density or volumetric energy density ($U_{density}$) is determined by dividing absolute ultrasound power with the volume (V) of the medium (cm^3 or mL) (eq. 1.4).

$$U_{density} = \frac{P}{V} \qquad (1.4)$$

Based on the frequency range, the applications of ultrasound in food analysis, quality control and processing can be divided into low energy (frequencies higher than 100 kHz at intensities below 1 W/cm^2,) and high energy (intensities higher than 1 W/cm^2 at frequencies between 20 kHz through to around 1 MHz) (AWAD et al., 2012).

At lower power ultrasound and wave frequency in the range of MHz, the changes in wave properties (velocity, attenuation, frequency, and spectrum) is used in diagnosis applications to characterize the medium. For some food systems, the speed of sound alone can be used to measure various physical properties, considered an accurate index for determining the concentration, composition and temperature of aqueous solutions such as sugar and alcohol solutions (CONTRERAS et al., 1992). The attenuation indicates the energy dissipation of the sound waves during transmission in a medium. Attenuation occurs more commonly in concentrated solutions and solid food systems (MICHAEL; LU; KATHRYN, 2005). Speed of sound has been shown to be sensitive to phase transitions in a system and successfully used to study lipid crystallization (MCCLEMENTS; POVEY; DICKINSON, 1993). It can also be used for obtain fluid velocity profiles through high-grade stainless steel pipes (KOTZÉ et al., 2015), and others.

When the applied power of ultrasound is higher, the acoustic waves could affect the medium generating interesting effects for industrial applications. The physical, mechanical, or chemical effects of power ultrasound (20 kHz through to around 1 MHz) are capable of altering material properties (e.g., disrupting the physical integrity, acceleration of certain chemical reactions) through generation of pressure, shear, and temperature gradients in the medium through which they propagate (RASTOGI, 2011). The acoustic cavitation plays an important role. Acoustic cavitation involves the rapid expansion and contraction of nano/micro bubbles of gas/vapour in a liquid subjected to the ultrasound. During the rapid changes of the bubble size, there is a net diffusion of gas/vapour molecules into the bubble causing it to grow to a resonance size range. Any further expansion under the effect of soundwaves can cause a sudden and instantaneous collapse and generate a hot spot with a temperature greater than 5000 K and pressure greater than 1000 atm. Such implosions occur at a number of sites within the liquid and as their numbers increase so does the physical shear forces and possibly the number of chemically active radicals also increases (BHASKARACHARYA; KENTISH; ASHOKKUMAR, 2009).

The ultrasound technology was studied in different food products, including meat products, vegetables and fruits, cereal products, aerated foods, honey, food gels, food proteins (AWAD et al., 2012). It is proposed and used in the food engineering and technology to accelerate many process such as dehydration (DENG; ZHAO, 2008), hydration (PATERO; AUGUSTO, 2015; MIANO; IBARZ; AUGUSTO, 2016), drying (GARCÍA-PÉREZ et al., 2006), freezing and thawing (KISSAM et al., 1982), tenderization of meat (RONCALÉS et al., 1993), to improve the emulsification (CUCHEVAL; CHOW, 2008; GAIKWAD; PANDIT, 2008), the physical properties (VERCET et al., 2002), and for modify the functional properties of different food proteins (YANJUN et al., 2014). There may be numerous advantages of using ultrasound for food processing such as effective mixing, increased mass transfer, reduced energy, reduced temperature and increased production rate. The ultrasound, in some studies, was proposed as an alternative method to thermal treatments without destroying nutrients of foods (FUJIKAWA; ITOH, 1996; MAWSON et al., 2011).

1.2   Physical properties and stability of beverages

The knowledge of the physical properties of food is fundamental in the food industry in order to design and evaluate each unit operations and the whole process. They influence the treatment received during the processing and they are good indicators of other properties and qualities of food (RAMOS; IBARZ, 1998). The food physical properties result of the responses of proteins, mono and polysaccharides, and lipids in aqueous media to different processing methods such as thermal processing, homogenization, and other physical treatments (GENOVESE; LOZANO; RAO, 2007). However, further than the food composition, its structure plays an important whole in the physical properties. The main physical categories of agro-food materials are: thermal, mechanical (rheological and textural), electrical, diffusional and optical (spectral and colour) (NESVADBA et al., 2004).

The fruit juices are composed by an insoluble phase (the dispersed phase or pulp, which is composed by fruit tissue cells and their fragments, cell walls and insoluble polymer clusters and chains) dispersed in a viscous solution (the serum, which is an aqueous solution of the intercellular components, such as soluble polysaccharides, sugars, salts and acids) (AUGUSTO; IBARZ; CRISTIANINI, 2012b). The main properties as consistency, sedimentation stability of fruit juices are associated with its flow behaviour (rheological responses). The rheological responses occur at macroscopic level. However, they are affected by the changes on its composition and structure at the molecular and microscopic level, which should lead to understanding the interrelationships between them (RAO; MCCLEMENTS, 2007).

Three kinds of forces coexist to various degrees in flowing dispersions: hydrodynamic, Brownian, and colloidal forces. The relative magnitude of these forces and, therefore, the bulk rheology depends on the particle size and particle volume fraction. Hydrodynamic forces dominate for particles larger than approximately 10 μm. When the particle volume fraction increases, the hydrodynamic interactions and the probability of collision between particles become important (QUEMADA; BERLI, 2002). On the other hand, Brownian motion and interparticle forces quickly equilibrate sub-nanometer-size dispersions (typically considered to be between 1 nm to 10 μm). Brownian motion promotes collisions between pairs of colloidal particles. However, the Brownian motion of each particle is hindered by the presence of the other ones (concentrated suspension), while interparticle forces determine if two colliding particles

aggregate or not. For particles in the intermediate range the flow behaviour is determined by a combination of hydrodynamic forces, Brownian motion, and interparticle forces (RUSSEL, 1980; QIN; ZAMAN, 2003)

Several foods are complex polydispersed systems that may contain microscopic particles that are >10 µm and up to approximately 100 µm (for example, fruit purees); some of them may be flocs or aggregates of colloidal particles. For such large particles, Brownian motion and interparticle forces are negligible compared to hydrodynamic forces. However, nonhydrodynamic parameters such as particle shape, particle size and size distribution, particle deformability, and liquid polarity could affect the structure and the resulting flow behaviour (VITALI; RAO, 1984; TANGLERTPAIBUL; RAO, 1987; TSAI; ZAMMOURI, 1988; AUGUSTO; VITALI, 2014).

In fact, there are many studies regarding the effect of different processes on the rheological properties of pulp and fruit juices, such as pineapple pulp (SILVA et al., 2010), jaboticaba pulp (SATO; CUNHA, 2009), acai pulp (TONON et al., 2009), orange juice (VITALI; RAO, 1984), peach juice (IBARZ et al., 1992; AUGUSTO et al., 2011), tomato juice (AUGUSTO; IBARZ; CRISTIANINI, 2012b), cloudy apple juice (GENOVESE; LOZANO, 2000, 2001, 2006), carrot juice (LIANG et al., 2006) and guava juice (ZAINAL et al., 2000). Furthermore, the other thermophysical properties was also studied for fruit juices, such as density, specific heat capacity, thermal conductivity, thermal diffusivity and freezing-point (RAMOS; IBARZ, 1998; TELIS-ROMERO et al., 1998; SHAMSUDIN; MOHAMED; YAMAN, 2005).

The juice physical stability normally refers to the appearance of the product and includes its propensity to separate into layers, the appearance of sediments, flocs or particulates as well any colour changes. Most of the physical changes that become apparent during the shelf life of a product are related to a chemical or biochemical reaction (presence of any residual pectolytic or other enzymes, the presence of dissolved oxygen and the temperature at which products are stored) and it may be more appropriate to this aspect of product stability as "physicochemical" rather than to being either strictly physical or chemical (KILCAST; SUBRAMANIAM, 2011). Changes in the appearance of a product are related to its intended nature for being either cloudy or clear. Different technologies was applied in order to improve their stability in cloudy juices such as acidification in carrot juice (SCHULTZ; BARRETT; DUNGAN, 2014) and in apple juice (ZHAO; ZONG; AN, 2008), enzymatic treatment in apple juice (OSZMIAŃSKI; WOJDYŁO; KOLNIAK, 2009), high pressure homogenization in

cashew apple juice (LEITE; AUGUSTO; CRISTIANINI, 2015) and in tomato juice (KUBO; AUGUSTO; CRISTIANINI, 2013).The main concern relating to stability changes of cloudy products is the separation of the product into sediment (which may be pulpy or compacted) and clear or almost clear supernatant layer (KILCAST; SUBRAMANIAM, 2011).

These changes reduce the consumer acceptance, so that many times is used packaging that prevents or limits the consumer from seeing all or part of the product until it is dispensed (KILCAST; SUBRAMANIAM, 2011). However, to improve the physical properties and to maintain the stability of juices during storage (to avoid the separation of solids and preventing sedimentation), the main approach is to add stabilizers (hydrocolloids) such as xanthan gum (XG) and carboxymethylcellulose (CMC) (GENOVESE; LOZANO, 2001; LIANG et al., 2006), which maintain solid materials like pulp in suspension. The aim of the stabilizers or mixtures of these is to maintain beverage homogeneity, but at the same time, providing low viscosity at moderate shear conditions. On the other hand, physical and mechanical technologies can be used to allow the juice stability, they are based principally in the structure modification, such as the use of high pressure homogenization (HPH) (LOPEZ-SANCHEZ et al., 2011; AUGUSTO; IBARZ; CRISTIANINI, 2012a; LEITE; AUGUSTO; CRISTIANINI, 2014, 2015; MOON; YOON, 2015) and the pulsed electrical fields (SCHILLING et al., 2008; ALTUNTAS et al., 2011), whose responses are changes in their rheological properties, sedimentation stability, settling velocity of particles, colour, turbidity and stability of bio-compounds.

However, despite the existence of different research on beverages, they simply describe the changes in the stability, physical properties and structural modification. Therefore, there is still the need to understand the relationship between these changes brought about by the used technology and its consequences. On the other hand, is the interest to obtain beverages with improved stability and organoleptic characteristics without the use of additives. These questions are pretended to resolve or improved through this work.

1.3   Ultrasound technology and physical properties

When ultrasound is applied to a solid–liquid system, the cavitation bubble collapse differs from that in a pure liquid system. Due to the proximity of a solid surface of particles, the bubbles often collapse asymmetrically. This has been shown to cause an inrush of fluid from the bubble towards the surface, a phenomenon referred to as micro-jetting. This effect leads to rapid heat and mass transfer at the solid surface as the boundary layer is disrupted. Micro-jetting can also destroy surface structures such as cell walls (BHASKARACHARYA; KENTISH; ASHOKKUMAR, 2009).

In fruit juices, a number of physical and mechanical effects can be obtained by processing it with ultrasound technology. Large particles are subject to surface erosion (by cavitation collapse in the surrounding liquid) or particle size reduction (due to fission through interparticle collision or the collapse of cavitation bubbles formed on the surface) (MASON; PANIWNYK; LORIMER, 1996). Further, with an increase in the ultrasonic power, there is an increase in the fraction of volume of the dispersed phase while the droplet size of the dispersed phase decrease (GAIKWAD; PANDIT, 2008). These effects can be confirmed by microscopic images and particle size measures (BI et al., 2015). Taking advantage of these effects, many studies have evaluated the use of the US technology as alternative for operations in fruit juices such as extraction (increase the extraction yield, and bioactive components (NGUYEN; LE; LE, 2012; BARBA et al., 2015), and as a potential technique to enhance the solution consistency of diluted products such as diluted avocado pure (BI et al., 2015), decrease the particle size and increase the apparent viscosity of tomato juice (WU et al., 2008). Since one of the compounds in fruit juices is the pectin, changes in its structure can be related with changes in the characteristics or properties of juices. Sonicated pectin solutions showed less turbidity, which can be related to the size, shape and volume fraction of the particles in the solution and showed a slower rate of gelation than the non-sonicated samples, probably due to chain length reduction of the pectin molecules reducing network formation (SESHADRI et al., 2003).

The effect of ultrasound processing to improve the stability of fruit juices during the storage time with respect at the acidity, pH, % soluble solids (^{o}Brix), electrical conductivity (EC), hunter colour values (L*, a* and b*), non-enzymatic browning, cloud value and bioactive compounds, was evaluated in previous works. In grapefruit juice, the ultrasonic processing showed no changes in pH, ^{o}Brix and acidity, while a

significant decrease in all the colour values (L*, a* and b*) and significant increase in EC, cloud value, total antioxidant capacity (TAC), and bioactive compounds were observed in all sonicated juice samples. The ultrasound processing promotes the stability, also during the storage, of all parameters mentioned above (AADIL et al., 2015). In apple juice, it contributed with the retention of ascorbic acid, total phenolic, flavonoids and flavonols (ABID et al., 2014a), as well as the cloud retention in orange juice (TIWARI et al., 2009b), stability of carrot juice during the storage (JABBAR et al., 2014), and also enhanced the pineapple juice colour and its stabilization along storage (COSTA et al., 2013).

Additionally, was demonstrated that juices processed with ultrasound, have better sensory acceptability than the pasteurized juices (ŠIMUNEK et al., 2013). However, a limited level of microbial inactivation was obtained by ultrasonic processing of juices, for example in orange juice a microbial growth was observed in the substrate following 14 days of storage at refrigeration temperature after ultrasonic process (VALERO et al., 2007). Therefore, to prevent the development of food-borne pathogens in juices it will be necessary to combine ultrasound with other processing methods with greater antimicrobial potency. In this regard, the use of ultrasound in combination with mild heating for industrial use is discussed.

1.4   Enzyme activity in vegetable products: importance and inactivation

Enzymes are globular proteins (macromolecules) that catalyse in a highly specific way the chemical reactions, taking place within a living cell. The binding and the catalytic activity occur in the active site of the enzyme, wherein all the necessary side chains of the amino acid residues are arranged. The active-site geometry is determined by the three-dimensional structure of the molecule (primary, secondary, tertiary, and quaternary structures) and is maintained by the noncovalent interactions within the enzyme (protein) molecule. Enzymes use all the forms of catalysis for attaining a high degree of efficiency (DEKKER, 2004b). Factors like pH, temperature, chemical agents (such as alcohol and urea), irradiation, mechanical shear stress, and hydrostatic pressure alter the active site and, consequently, affect the performance of enzymes (DEKKER, 2004a).

The peroxidase (POD, EC 1.11.1.7) and the polyphenol oxidase (PPO, EC 1.14.18.1) are two important enzymes in many fruits and vegetables. Their residual activity is detrimental to the quality of processed products of fruits and vegetables resulting in effects such as browning, off flavour and loss of vitamins. Therefore, the inactivation of POD and PPO in the processing of fruits and vegetables is a major quality indicator of processed fruits and vegetables. In coconut water, the enzymes are naturally present and cause sensory and physicochemical deteriorating quality.

Peroxidases are enzymes whose primary function is the oxidation of phenolic residues at the expense of hydrogen peroxide (DAVID, 2000). The peroxidation reaction is the most important one. The mechanism of reaction essentially involves an oxidative action by way of an initial formation of a complex intermediate with a hydrogen acceptor. The transfer of hydrogen from a donor substrate results in a second complex intermediate before the regeneration of the POD enzyme and formation of a reaction product. The hydrogen donor complexes and two univalent oxidation intermediate steps occur (OLUSOLA, 2002):

$$POD + H_2O_2 \rightarrow Complex\ I$$
$$Complex\ I + AH_2 \rightarrow Complex\ II + AH$$
$$Complex\ II + AH \rightarrow POD + A$$
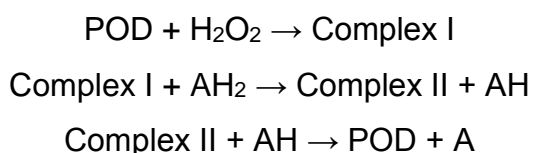
Figure 1.2 – Peroxidase mechanism reaction (OLUSOLA, 2002)

Where AH_2 is the hydrogen donor in the reduced form, and A is the hydrogen donor in the oxidized form. The ability of POD to contribute to the enzymatic browning is related to its affinity to accept a wide range of hydrogen donors, such as polyphenols. They are able to oxidize catechins, hydoxycinnamic acid derivatives and flavonoids. Two possible mechanisms are proposed for POD catalyzed browning reactions. One involves the generation of H_2O_2 during the oxidation of some phenolic compounds that is used as in a normal peroxidatic action to further oxidize the phenol, while the second involves the use of quinonic forms as substrate by POD. Both mechanisms indicate that the presence of polyphenol oxidase enzyme would enhance POD-mediated browning reactions (OLUSOLA, 2002).

The regeneration of enzymatic activity after thermal denaturation is unusual for enzymes, in general, although it is a well-recognized property of peroxidases. The ability of peroxidases to regenerate after thermal denaturation varies not only between different source species, but also between the isoenzymes within a single variety (DAVID, 2000).

1.4.1 Coconut water

The coconut water, also called as the liquid albumen, is obtained inside the fruit of the coconut (*Cocos nucifera L.*). The value of gross agricultural production of coconuts updated at 2013 is represented mainly by the following countries: Indonesia, Philippines, India, Brazil and Papua New Guinea with 29.3%, 24.6%, 19.1%, 4% and 2% of the world production, respectively (FAO, 2015). The demand for coconut water production has grown in the last years due to different uses in a traditional medicine, a microbiological growth medium, a ceremonial gift, as vinegar or wine production, but, specially, as a tropical beverage (DEBMANDAL; MANDAL, 2011; PRADES et al., 2012). Coconut water is considered as a sports refreshing drink because it contains an adequate proportion of salts and minerals such as potassium, sodium, chloride, magnesium and some sugars. In fact, it does not have any other natural product with similar effect (NOEL, 2008). It is estimated that about 10% of the coconut Brazilian production is intended for processing coconut water, for its filling and subsequent marketing, which represents a volume of 70 million litters (PENHA; CABRAL; MATTA, 2005). These various uses are possible due to its sensory properties and to the original biochemical composition.

The great variability in the composition of coconut water is influenced not only by the interaction of the variety and maturity stages, but also by the composition of the soil where the plant is grown (DE CARVALHO et al., 2006). The pH of the coconut water varies with the age of the fruit, and when the age of 5 months, the pH is around 4.7 to 4.8, rising above 5 to the end of fruit growth (ARAGÃO; ISBERNER; CRUZ, 2001). Its basic composition according to Brazilian table of food composition is shown in Table 1.1 (NEPA-UNICAMP, 2011).

Table 1.1 – Average composition of coconut water

Composition/100 g		Quantity
Energy value	(kcal)	21.5
Protein	(g)	0
Lipids	(g)	0
Cholesterol	(mg)	NA
Carbohydrate	(g)	5.3
Dietary Fibre	(g)	0.13
Ash	(g)	0.5
Calcium	(mg)	18.8
Magnesium	(mg)	5.2
Manganese	(mg)	0.3
Phosphorus	(mg)	3.8
Iron	(mg)	Tr
Sodium	(mg)	1.8
Potassium	(mg)	161.7
Copper	(mg)	Tr
Zinc	(mg)	Tr
Retinol	(mcg)	NA
Thiamine	(mg)	<0.1
Riboflavin	(mg)	Tr
Pyridoxine	(mg)	Tr
Niacin	(mg)	Tr
Ascorbic acid	(mg)	2.4

Source: Brazilian table of food composition (NEPA-UNICAMP, 2011)
Abbreviations: NA: not applicable; Tr: trace

In addition, other authors describe additional interesting compounds in coconut water. It contains free amino acids like serine, glycine, histidine, tyrosine, phenylalanine, isoleucine and leucine (OVALLES et al., 2002). Vitamins of the B-complex, such as B3 and nicotinic acid (0.64 g/mL), B5 pantothenic acid (0.52 g/mL), riboflavin B2 (<0.01 g/mL), biotin (0.02 g/mL), folic acid (0.003 g/mL), and trace amounts of thiamine (B1) and pyridoxine (B6), in its composition are also phytohormonas, enzymes (phosphatase, catalase, dehydrogenase, diastase,

peroxidase, polyphenoloxidase, RNA polymerases) and growth promoting factors (YONG et al., 2009; DEBMANDAL; MANDAL, 2011; PRADES et al., 2012).

The presence of polyphenol oxidase and peroxidase enzymes in green coconut water is considered a very important factor due to the resulted undesirable changes, as the development of pinkish and/or dark brown colour. The pink discoloration in coconut water is a result of the initial enzymatic action of polyphenol oxidase (PPO) on the phenolic substrates which are naturally present in the product. Activity of the enzyme is further enhanced by metal contamination such as copper and exposure of the product to high temperature during its processing and storage. There is evidence that the enzyme activity occurs with fullness in fruits aged five to seven months, decreasing with ripening (PENHA; CABRAL; MATTA, 2005).

Traditionally, the coconut water is marketed in the fruit itself, a practice that involves issues related to transportation, storage and perishability of the product. In order to allow its use in locations outside of the producing regions, its industrialization is fundamental to reduce the volume and weight transported and consequently reduce costs and increase its shelf life and market (NAOZUKA et al., 2004; DE CARVALHO et al., 2006).

The basic forms of commercialization of coconuts include the minimal processing (green tender coconuts) and the cooling. The minimum processing consists of dipping partially husked nuts in a solution of 0.5% citric acid and 0.5% of potassium metabisulphite for 3 min, to prevent the coconut skin browning. The final product, packaged with PP, PE, PVC or paraffin based coating can be stored for up to 24 days under refrigeration temperatures (PRADES et al., 2012). During that, microbiological and physicochemical (as modification of the colour) changes are likely to occur (PENHA; CABRAL; MATTA, 2005).

The shelf life of coconut water after their extraction depends on the applied preservation methods. These methods should aim to inhibit the enzyme activity, ensure the microbiological quality and maintaining the original sensory characteristics after opening the walnut (PENHA; CABRAL; MATTA, 2005). Usually, the methods involve thermal processing, filtration, sugar adjustment, pH and total solids, concentration by reverse osmosis (MANJUNATHA; RAJU, 2013), added preservatives, carbonation, etc., and various combinations of methods for preservation (DE CARVALHO et al., 2006). For this, it is essential to optimize the processing time and reduce the exposure to the air. Depending on the type of used processing, different

products can be obtained, such as the cooled coconut water, frozen coconut water, pasteurized and frozen coconut water, sterilized coconut water, ultrafiltered coconut water bottled aseptically, and coconut water with additives (PENHA; CABRAL; MATTA, 2005). A brief overview of the principal processes and technologies used in order to increase the useful life of green coconut water is presented as follows.

During the thermal process, filtered coconut water is subjected to pasteurization or sterilization. Temperatures in the range of 92-95 °C, for 20 to 40 seconds, is used during pasteurization. At this stage, the heat-sensitive pathogens and microorganisms are inactivated. For sterilization by ultra-high temperature (UHT), are used temperatures in the range of 140-150 °C for 1 to 5 seconds (PENHA; CABRAL; MATTA, 2005). The thermal processing for inactivation of polyphenol oxidase and peroxidase enzymes in green coconut water is effective only above 90 °C for more than 300 s (CAMPOS et al., 1996). For example, at 139 °C for 10 s combined with 200 mg/L of ascorbic acid, PPO was inactivated until undetectable activity, whereas POD was still active at 40% of its original level (ABREU; FARIA, 2007). The problem is that above 90 °C and from 90 seconds exposure of the product at this conditions already occurs sensory and nutritional problems in the coconut water such as changes in aroma and flavour (PENHA; CABRAL; MATTA, 2005), decreases the total phenolic and soluble solids (NAKANO et al., 2011), formation of compounds with a toasted and malty aroma with sensory differences perceived between thermally processed and fresh coconut water (CAPPELLETTI et al., 2015). In fact, the thermal process of coconut water is commercially used in combination with additives as ascorbic acid, sodium metabisulphite, sodium benzoate among others, although its negative impact on sensorial and nutritional properties makes it necessary to study alternatives.

On the other and, the coconut water freezing is performed in cold rooms at temperatures of - 40 °C, the main disadvantage is that the product requires cold chain until the moment of consumption and must be kept frozen. The temperature recommended for its storage in frigorific chambe8rs is between -18 °C and -20 °C. The shelf life of these products range from three to six months (PENHA; CABRAL; MATTA, 2005).

For microfiltration, a 0.1 µm pore size membrane was evaluated and for the ultrafiltration 100, 50 and 20 kDa molecular weight cut off membranes were evaluated (MAGALHÃES et al., 2005). The permeate fraction can be considered as cold pasteurized, where enzymes and microorganisms are retained by the membrane,

using polymeric or ceramic membranes in semi-pilot scale (PENHA; CABRAL; MATTA, 2005; DAS PURKAYASTHA et al., 2012). The product obtained is acceptable for microbial, sensory and physicochemical properties for 46 days under 4 °C refrigeration conditions (MAHNOT et al., 2014). However, it was demonstrated that the filtration reduced different nutrients of the coconut water like fat, ash, total sugar, reducing sugar, minerals and protein. Also; the flavour and overall acceptability decreased about 9 and 11%, respectively (REDDY; DAS; DAS, 2007). Further, the consumer evaluation indicated that the reduction of soluble solids (10%), total phenolic and other compounds may cause its loss of flavour, decreasing its acceptability scores (NAKANO et al., 2011).

The production of coconut water with preservatives and carbon dioxide addition were also studied, as standardizing with 200 mg/L of ascorbic acid, clarifying, adding potassium sorbate and sodium metabisulphite, pasteurizing at 90 °C for 30 seconds, and finally carbonating it with 2 to 3 volumes of CO_2 (PEREIRA; FARIA; PINTO, 2013). The addition of CO_2 to the coconut water, besides giving a more refreshing sensation to the beverage, also contributed to its conservation because it helped to reduce the pH value of the product, as well as reducing the amount of dissolved oxygen (PEREIRA; FARIA; PINTO, 2013). However, the overall acceptability decreased with the increase in ascorbic acid concentration due to higher acidic flavour formation (DAS PURKAYASTHA et al., 2012)

However, while these technologies may be effective for microbial inactivation and product safety assurance, beyond mentioning the current consumer preference for natural products without additives, the product obtained still needs more stability. At respect, since thermal processing offers microbiological stability, however the enzymatic stability is still a challenge, the ultrasound technology is proposed here as an alternative, which was not yet studied for the enzyme inactivation of coconut water, with expectations for improving the conventional thermal processing.

1.4.2 Technologies for enzyme inactivation

Traditional thermal methods such as sterilization, pasteurization, precooking or blanching are the methods most known and used by the food industry for the inactivation of enzymes. However, other alternative technologies are gaining importance recently such as High Pressure Carbon Dioxide (HPCD) (LIU et al., 2008)

and Pulsed Electric Fields (PEF) where the decrease in the enzymatic activity (71% and 68%, for PPO and POD, respectively) was significantly higher than that recorded in juice processed by conventional mild pasteurization at 72 °C for a holding time of 26 s (46% and 48%, respectively) (RIENER et al., 2008; SCHILLING et al., 2008). Table 1.2 shows different technologies investigated in the inactivation of polyphenol oxidase and peroxidase for different vegetable products.

Furthermore, different studies have been performed in coconut water in order to inactivate the PPO and POD enzymes (Table 1.3). However, the ultrasound technology has not been studied yet. The technologies used in the manufacture of commercial coconut water are the conventional thermal process used alone or in combination with ultrafiltration, additives such as ascorbic acid, potassium sorbate and sodium metabisulfite. As well as other technologies such as microfiltration and ultrafiltration, processing with microwave and ultraviolet radiation. All the studied methods have demonstrated an effective reduction of the enzyme activities. However, the enzymes naturally present in coconut water showed a higher resistance when compared to those added to the sterilized medium or other simulated solutions reported in the literature (MATSUI et al., 2007; AUGUSTO et al., 2015). Probably the enzymes naturally present in coconut water, are more adapted at the medium or matrix and have more resistant enzyme fractions (isoenzymes).

Table 1.2 – Technologies used for enzyme (POD and PPO) inactivation

Method	Sample	Enzyme	Author
TT and HP Processing	blueberry	PPO	(TEREFE et al., 2015)
TT and HP Processing	pear, apple and strawberry purees	PPO	(SULAIMAN et al., 2015b)
TT and HP Processing	Pineapple puree	PPO, POD	(CHAKRABORTY; RAO; MISHRA, 2015)
TT and HP Processing	strawberry	PPO	(DALMADI et al., 2006)
TT	Apple enzyme extract	PPO, POD	(VALDERRAMA; FABIANE; CLEMENTE, 2001)
TT and MW.	Carrot	POD	(SOYSAL; SÖYLEMEZ, 2005)
TT and PEF	Apple juice	PPO, POD	(RIENER et al., 2008; SCHILLING et al., 2008)
PEF	Commercial enzyme solution	PPO, POD	(ZHONG et al., 2007)
PEF	Grape juice	PPO, POD	(MARSELLÉS-FONTANET; MARTÍN-BELLOSO, 2007)
HHP and DPCD	Commercial enzyme from mushroom	PPO	(DUONG; BALABAN; PERERA, 2015)
HHP and DPCD	Feijoa puree	PME, PPO, POD	(DUONG; BALABAN, 2014)
SCD	Cloudy apple juice	PPO	(GUI et al., 2007)
HPCD	Red beet	PPO, POD	(LIU et al., 2008)
HP Processing	Mango	PME, PPO, POD	(KAUSHIK; NADELLA; RAO, 2015)
UV	Commercial enzyme solution	PPO	(FALGUERA et al., 2012; FALGUERA et al., 2013)

Table 1.3 – Technologies used in coconut water for the enzyme (POD and PPO) inactivation

Method	Processing conditions	Author
UV*	UV (400 W, WL: 250 - 740 nm), For 1, 2, 3, 6, 9, 12, 15, 20, 25, 30, 40, 50 and 60 min	(AUGUSTO et al., 2015)
TT	TT at 80, 85, 90 and 95 °C for 2.5, 5, 10 and 15 min.	(TAN et al., 2014)
TT with additives	TT at 90 °C for 30 s, Potassium sorbate (from 0 to 500 mg/L), sodium metabisulphite (from 0 to 100 mg/L)	(PEREIRA; FARIA; PINTO, 2013)
TT	TT at 75, 85 e 95 °C for 1min to 30 min	(FONTAN et al., 2012)
TT	TT at 75.6, 79.4 and 86.9 °C with immersion times from 19 to 502 s.	(MURASAKI-ALIBERTI et al., 2009)
TT with AA	TT(UHT) for 138 - 144 °C and AA (0 - 200 mg/L)	(ABREU; FARIA, 2007)
MW	MW at 52.5 °C and 92.9 °C	(MATSUI et al., 2008)
MW*	Batch MW at 60 - 100 °C	(MATSUI et al., 2007)
MF with L-AA	MF (0.45 µm pore size, 47 mm diameter) with L-AA (25 mg/100 ml)	(DAS PURKAYASTHA et al., 2012)
UF and TT	UF (20 kDa, at 5 bar and 15 °C) TT: 96 °C for 20 s.	(NAKANO et al., 2011)
MF and UF	0.1µm, and cut off of 100, 50 and 20 kDa	(MAGALHÃES et al., 2005)

*coconut water model or simulated solutions

1.4.2.1 Thermal processing and enzyme inactivation

Thermal inactivation of POD can be either by dissociation of the prosthetic (heme) group from the holoenzyme (active enzyme system), conformational changes in protein or by modification or degradation of the prosthetic group. A large decrease in activity is observed during the initial stages of a given thermal process, but after the rate of inactivation changes to a much slower process. The biphasic nature of thermal inactivation (nonlinear) is related to the presence of isozymes with differing stabilities in vegetables (OLUSOLA, 2002). However, in microwave treatment it was observed a biphasic and monophasic behaviour depending on the amount of energy applied (biphasic behaviour of enzyme inactivation was observed for the microwave treatment at 70 and 210 W, whereas at 350 and 700 W microwave powers enzyme inactivation

was monophasic). Microwave heating was found to be more effective for enzyme inactivation than the conventional thermal process, because the microwave penetration promotes a fast heating rate and it also has a non-thermal effect on enzyme inactivation attributable to the intrinsic nature of microwaves (SOYSAL; SÖYLEMEZ, 2005; MATSUI et al., 2007, 2008).

An unusual phenomenon observed in some products is the enzyme reactivation after thermal processing. At a high temperature, the protein molecule will unfold, but if nothing else happens, it will probably refold after cooling and thereby regain its enzyme activity. This means that the unfolded molecule must undergo a reaction that prevents it from refolding into its native conformation. In thermal methods applied in natural coconut water, it has been found the presence of residual isoenzymes with thermal resistance which could interfere in the product stabilization (NAKANO et al., 2011).

The polyphenoloxidase and peroxidase activities were not significant in any of the samples during the storage time, indicating that the thermal processing (90 °C for 30 s) applied to the samples was sufficient to inactivate them (PEREIRA; FARIA; PINTO, 2013). While it is true that at high temperatures inactivation of enzymes (POD, PME and PPO) and microorganisms (total plate count, yeast and mould) is achieved in conventional pasteurization at 95 °C for the appropriated time, the principal inconvenience of this treatment is that showed highest losses of ascorbic acid, total phenols, flavonoids and antioxidant capacity (SAEEDUDDIN et al., 2015). Therefore, it highlights the need for investigate alternative technologies that ensure the microbial, enzymatic and nutritional stability.

1.4.2.2 Ultrasonic processing and enzyme inactivation

The ultrasonic inactivation mechanisms are specific to the enzyme under investigation and depend on its amino acid composition and the conformational structure (ÖZBEK; ÜLGEN, 2000). The formation and disintegration of bubbles induce cavitation in the sample which is a physical phenomenon in enzyme inactivation (ERCAN; SOYSAL, 2011). It has also been reported that one of the major factors in enzyme inactivation is the severe rise in temperature (5000 K) and pressure (1000 atm) in a localized ultrasound generating area resulting in high shear rates and generating strong micro-streaming, which can contribute for enzyme and microbial

inactivation (VERCET et al., 2001). Further, during the ultrasound processing, free radicals are produced in sonolysis of water molecules ($H_2O \rightarrow OH^- + H^+$), these hydrogen peroxide and hydroxyl-free radicals determine a diffuse aggregation and a loss of its enzymatic activity (TEREFE et al., 2009). For example, in porcine fumarase processed with ultrasound (US) at 38 kHz, aggregation is caused by the formation of intermolecular bisulfide bridges, originated by the oxidation of cysteine residues, together with a diffuse increase in β-turn in the protein's secondary structure (BARTERI et al., 2004). It is difficult to identify the specific enzyme inactivation mechanism during sonication, which could be due to a singular or combination of several chemical and physical effects occurring simultaneously (RAWSON et al., 2011). However, a possibility is related to the conformation changes in the tertiary structure, as in the active site three-dimensional structure affecting the enzyme–substrate interaction (CRUZ; VIEIRA; SILVA, 2006). The splitting of prosthetic group of hemoproteins has been proven in an ultrasonic field that can contribute to denaturation (WEISSLER, 1960).

There are many studies of the application of ultrasound for the enzyme inactivation, such as for lipases, proteases, peroxidase (POD), polyphenoloxidase (PPO), polygalacturonase (PG), pectinesterase, pectinmethylesterase (PME), ascorbate peroxidase (APx). However, long processing times and high intensities are required to achieve inactivation (TIWARI et al., 2009a; COSTA et al., 2013; HUANG et al., 2015). Further, the ultrasound treatments with a lower power level, induce the enzyme activity and phenolic production as part of plant stress responses to a mechanical stimulus (WU; LIN, 2002). In fact, partial inactivation effects (i.e. the enzyme inactivation is not achieved completely) were reported (SILVA et al., 2015), or a relative inactivation under specific operating conditions (time, ultrasound intensity, temperature) cause an increase or decrease in enzyme activity (FONTELES et al., 2012; ENGMANN et al., 2014). Such as, an increase in enzyme activity was observed for POD with increasing processing time and for PPO at higher temperatures (60 °C) (SILVA et al., 2015). On the other hand, PPO inactivation was reduced as ultrasonic frequency and treatment time were increased, indicating an inverse relationship. The plausible reason for increased activity may be due to activation of latent PPO isoenzymes by the ultrasonic process (ENGMANN et al., 2014), also could be related with the change of conformation of the enzyme to a higher enzyme–substrate

interaction, and consequently to an optimal stage of consumption of the substrate (CRUZ; VIEIRA; SILVA, 2006).

Since in general the ultrasound alone cannot inactivate enzymes to a desired level, it must be combined with other technologies in order to reach the desirable goal. For example, in order to enhance the effect of enzyme inactivation, ultrasound (US) is used in combination with other technologies such as high hydrostatic pressure (HHP) (mano-sonication (MS)), ultraviolet radiation (photo-sonication (PS)), temperature (thermo-sonication (TS)) or by combining them with the temperature being obtained mano-thermo-sonication (MTS), photo-thermo-sonication (PTS), and in combination with additives such as ascorbic acid. Table 1.4 shows the ultrasound applications alone or in combination with other technologies under different conditions and their effects on the enzyme inactivation of vegetable products.

Table 1.4 – Ultrasound applications alone or in combination with other technologies and their enzymatic inactivation effects

(Continued)

Sample	Processing conditions	Enzyme and effect	Author
Pear, apple, strawberry purees	US (24 kHz, 10 min, 32 ± 3.6 °C), and TS (52 ± 3.1 °C and 72 ± 1.2 °C) at 1.3 W/g. The TS was compared to TT between 55 °C and 80 °C.	PPO (US increased the TS inactivation rate constant)	(SULAIMAN et al., 2015a)
Pear juice	TS (750 W, 20 kHz and amplitude 70%) at 25, 45 and 65 °C for 10 min, TT at 65 °C for 10 min and 95 °C for 2 min.	PPO, PME, POD (TS at 65 °C; highest inactivation)	(SAEEDUDDIN et al., 2015)
Apple cubes	US from 55 to 3300 W/L, at 23 °C to 60 °C for 5 to 20 min.	PPO, POD (Partial inactivation)	(SILVA et al., 2015)
Mulberry juice	US (60 W) at 22, 24 and 26 kHz, for 10, 20 and 30 min each one.	PPO, POD (Relative inactivation)	(ENGMANN et al., 2014)
Orange juice	TS (at 80 W) for 21.80 min at 75 °C and 9.8 min at 63 °C	PME (Entirely inactivate)	(KOSHANI et al., 2014)
Apple juice	US (25 kHz and 70% amplitude) at 20 °C for 60 min with HHP at 250, 350 and 450 MPa for 10 min.	PPO, PME, POD (US-HHP450; highest inactivation)	(ABID et al., 2014b)

(Conclusion)

Sample	Processing conditions	Enzyme and effect	Author
Mushroom	TT and TS (0.43 W/mL, 25 kHz) from 55 to 75 °C for 30 to 4 min.	PPO (D values TT>TS, E_a and z-values TT<TS)	(CHENG; ZHANG; ADHIKARI, 2013)
Fresh-cut apple	US (40 kHz), AA (1%), UA(US+AA)	PPO, POD (UA inactivate, US and AA effect inverse).	(JANG; MOON, 2011)
Watercress (*Nasturtium officinale*)	TT (40 to 92.5 °C), TS (20kHz)	POD (at T<80 °C increased the activity; at T>80.2 °C, inactivation)	(CRUZ; VIEIRA; SILVA, 2006)
Tomato juice	TT and TS (40 W, 20 kHz), at 50 - 75 °C.	PG, PME (Inactivation at T>50 °C)	(TEREFE et al., 2009)
Apple juice	TT (90 °C for 1 min.), PTS (2 and 4 UV lamps with 4.4 mW/cm^2 + US, 24kHz) at 40, 50 and 60 °C for 5 and 10 min.	PPO (at > T, > inactivation)	(BASLAR; ERTUGAY, 2013)
Orange juice	US (0.42, 0.47, 0.61, 0.79 and 1.05 W/mL) for 0, 2, 4, 6, 8 and 10 min.	PME (At highest level treatment, 62% inactivation).	(TIWARI et al., 2009a)
Tomato	US (0.004–0.020 mg.L/min), TT and TS at 50, 61, and 72 °C.	PME (inactivation was more pronounced at < temperatures)	(RAVIYAN; ZHANG; FENG, 2005)
Pineapple Juice	US at 0.67, 2, and 3.3 W/mL for 2-10 min.	PPO (20% activity reduction at longer exposure and higher intensity)	(COSTA et al., 2013)
Cantaloupe melon juice	US at 75 to 376 W/cm^2 for 2 to 10 min.	PPO, POD, APx (POD activity increase at low processing time; PPO and APx decrease in all treatments)	(FONTELES et al., 2012)
Satsuma mandarin	US (20 kHz, 200 W for 10, 20, 30, 40 min) and (100, 200, 400 W for 30 min),	PPO (At highest level treatment, > PPO inactivation)	(HUANG et al., 2015)

Thermo-sonication (TS), is an interesting technology because the heat and mechanical forces in the ultrasound pasteurization have a synergistic effect on enzyme inactivation and the required results were achieved at lower temperature (SAEEDUDDIN et al., 2015). It was observed a higher rate of inactivation for combined ultrasound and thermal process compared to the thermal process alone. On the other hand, during the thermal inactivation of peroxidase from different sources, a deviation of first-order kinetics (biphasic inactivation) is frequently observed (LÓPEZ et al., 1994). This phenomenon is generally accepted to be due to the presence of enzyme aggregates with different stabilities (isozymes) (CHENG; ZHANG; ADHIKARI, 2013). However, with ultrasonic application, the monophasic inactivation of peroxidase could be attributed to the dissociation effect of ultrasonic waves on aggregates (LÓPEZ et al., 1994).

1.5   Enzymatic inactivation kinetics

The enzyme inactivation kinetics are evaluated and modelled considering different possibilities of inactivation, as described during ultraviolet irradiation (FALGUERA et al., 2012; FALGUERA et al., 2013; AUGUSTO et al., 2015), high-pressure carbon dioxide (LIU et al., 2008), pulsed electric fields (ZHONG et al., 2007), high pressure and thermal processing (ANN et al., 2005; AGUIAR; YAMASHITA; GUT, 2012; SULAIMAN et al., 2015b).

The simplest behaviour for the enzyme inactivation mechanism describes the passage of the native form of the enzyme to a single inactive state due to denaturation (eq. 1.5). It considers only one enzymatic portion to be inactivated, and it can be described by the first order kinetics. Therefore $\frac{A_t}{A_0}$ is the residual enzyme activity where A_0 is the enzyme activity for $t = 0$.

$$\frac{A_t}{A_0} = \mathrm{e}^{(-k.t)} \tag{1.5}$$

However, different behaviours can be observed during the enzyme inactivation process. Other mechanisms with different enzyme portions and/or intermediate states results, in more complex multi component inactivation kinetics, characterized by the

enzymatic resistance presented to the technology used (FUJIKAWA; ITOH, 1996; MURASAKI-ALIBERTI et al., 2009). Two enzymatic portions are frequently observed in the inactivation studies, as PPO and POD thermo-inactivation in coconut water (MURASAKI-ALIBERTI et al., 2009; PRADES et al., 2012) and photo-inactivation (AUGUSTO et al., 2015). This reaction involves sensitive and resistant portions. This approach considers that existing fractions are autonomous and that the inactivation of each is independently. The inactivation of the two portions follows a first order kinetics (eq. 1.6).

$$\frac{A_t}{A_0} = \alpha . \mathrm{e}^{(-k_S . t)} + (1 - \alpha) . \mathrm{e}^{(-k_R . t)} \qquad (1.6)$$

Where k_S is the constant of inactivation of the sensitive portion, k_R is the constant of inactivation of the resistant portion and α is the relative resistance of the sensible enzyme portion.

When a fraction of the enzyme is not destroyed after prolonged treatments (A_∞), the enzyme inactivation kinetics follow a fractional conversion model (eq. 1.7) (TIWARI et al., 2008; TEREFE et al., 2009)

$$A_t = A_\infty + (A_0 - A_\infty) . \mathrm{e}^{-k . t} \qquad (1.7)$$

Where A_∞ is the residual activity after prolonged treatment time, i.e. the activity of the stable fraction. It is indicated that when $A_\infty \to 0$, the eq. (1.7) tends to a first-order kinetic model (eq. 1.5).

In other cases, as described by Giner-Seguí et al. (2006) the enzyme inactivation under Pulsed Electric Fields is supposed to occur in two consecutive irreversible first order steps with the presence of intermediate active forms of the enzyme between the native and the completely inactivated enzyme. After performing the appropriate balances described by Giner-Seguí et al. (2006), this behaviour is described by the following equation.

$$\frac{A_t}{A_0} = \mathrm{e}^{-k_1 . t} - \frac{k_1 . \Lambda}{(k_1 - k_2)} . (e^{-k_1 . t} - \mathrm{e}^{-k_2 . t}) \qquad (1.8)$$

Where the parameter Λ describes the ratio between the activities of the native and intermediate states.

As described above the model most used is the classical first order or its derivations, which describes a simplest behaviour for the enzyme inactivation mechanism. However, there are indications that the inactivation is not a simple mechanism, since nonlinear trends are often observed experimentally in microbial and enzyme inactivation (SERMENT-MORENO et al., 2014). Further, plants naturally presents multiple forms of the same type of enzyme, that catalyse the same reaction, but the individual isozymes may differ markedly in physicochemical and kinetic properties (SHANNON; KAY; LEW, 1966). Therefore, taking into account the complexity of enzyme inactivation, where the number of equivalent mechanisms increases rapidly during the process (POLAKOVIČ; VRÁBEL, 1996), many models have been developed as alternatives to linear inactivation kinetics, such as Weibull Model, Weibull Biphasic Model, Log-Logistic Model, among others (SERMENT-MORENO et al., 2014).

Even though the Weibull distribution (eq. 1.9) was used in engineering science to predict the time of failure $F(t)$ of an electronic or mechanical system (eq. 1.9) (WEIBULL, 1951; SMITH, 1991). It was adapted by PELEG and COLE (1998) to model microbial survival curves, it has been used to describe numerous inactivation kinetics because it is considered a simple (only 2 parameters), flexible and realistic model (eq. 1.10).

$$F(t) = \exp\left[\left(-\frac{t}{p}\right)^q\right] \tag{1.9}$$

$$\frac{A_t}{A_0} = \exp(-b.\,t^n) \tag{1.10}$$

Thus, the residual microbial/enzyme activity curve (eq. 1.10) can be interpreted as a cumulative function of the distribution that dictates the treatment time at which the microorganism or enzyme will fail to resist and result in inactivation. The parameter b determines the scale of the curve and n determines the shape of the survival curve,

where $n < 1$ denotes upward concavity and $n > 1$ represents a downward concavity while $n = 1$ would be a unique case corresponding to linear or first-order kinetics (SERMENT-MORENO et al., 2014). The n value can be used to interpret the population inactivation resistance: (a) homogenous ($n = 1$), (b) tailing or increasing resistance ($n < 1$), or (c) decreasing resistance as a result of accumulated damage to the population ($n > 1$) (PELEG; COLE, 1998; VAN BOEKEL, 2002)

Enzymatic inactivation kinetic during the thermal processing

During the thermal process the enzyme inactivation kinetics can be modelled according the eq. (1.5), (1.6), (1.7), (1.8) and (1.9). These are commonly described using the parameters D_T value and z value. The D_T value is the time required for a 90% reduction of the microorganism population or enzymatic activity under isothermal conditions. The effect of temperature on the D_T value is expressed as a z value, which is the temperature increase that reduces it by 90% (AUGUSTO; TRIBST; CRISTIANINI, 2014). The parameters determination and modelling of the enzymatic inactivation depends of the type of the thermal process, basically if it is isothermal or non-isothermal process (TAJCHAKAVIT; RAMASWAMY, 1997).

Under isothermal processing

The eq. (1.11) presents the model for enzymatic inactivation under isothermal processing at temperature T for a holding time t.

$$\log \left(\frac{A_t}{A_0} \right) = -\frac{t}{D_T} \tag{1.11}$$

For first order reactions, the D_T value is related to the first order reaction rate constant, k. It can be mathematically expressed in terms of k value as given by (eq. 1.12). While the z value is obtained from the negative reciprocal of the slope of $\log(D)$ versus temperature curve.

$$D_T = \frac{\ln(10)}{k} \tag{1.12}$$

Under non-isothermal processing

In most thermal processing situations, food products are subjected to non-isothermal processing. The lethality (L) accumulated at the cold spot is generally obtained by integration of the lethal effects of the temperature profile during the come-up, hold and cooling periods (TAJCHAKAVIT; RAMASWAMY, 1997). The accumulated lethality (L) is expressed as the effective or equivalent holding time (t_{equiv}), which is the isothermal holding time at T_{ref} results in the same 'lethal effect' $\frac{A_t}{A_0}$, can be obtained from the time-temperature history of the product $T_{(t)}$ through eq. (1.13), which can be evaluated numerically using the trapezoidal method (MURASAKI-ALIBERTI et al., 2009).

$$t_{equiv} = \int_0^\infty 10^{\wedge}(\frac{T_{(t)} - T_{ref}}{z})dt \qquad (1.13)$$

According to Tajchakavit and Ramaswamy (1997), since there is no specific isothermal period, any temperature within the range of study could be used as a reference temperature. However, for each experiment, the highest temperature (T_{max}) can be considered as reference temperature (T_{ref}) (MURASAKI-ALIBERTI et al., 2009).

Computation of the effective time or lethality requires data on z-value which needs to be obtained from a regression of $\log(D)$ vs temperature. For this purpose, first estimates of the D_T associated at the various temperatures (T) are obtained using uncorrected heating times assuming isothermal heating conditions. These values are used to calculate the z value by regression of $\log(D)$ vs T as the negative reciprocal slope. After this z value is used to calculate the effective heating time under each condition using the eq. (1.13) from which corrected D_T values are calculated. These D_T values are then used to get a new z value which is then used again to get more precise values of effective heating times and used to recalculate the D_T values and subsequently another z. The process generally is repeated for the convergence of D_T and z values (TAJCHAKAVIT; RAMASWAMY, 1997). The predicted residual activity for each experimental run can be calculated from the numerical evaluation of the integral in the eq. (1.14) (PELEG; PENCHINA; COLE, 2001; AGUIAR; YAMASHITA; GUT, 2012). The eq. (1.14) shows the predicted residual activity only for the simple

first-order kinetics eq. (1.5), for the another cases it is necessary to replace the eq. (1.13) in the equations (1.5), (1.6), (1.7), (1.8) and (1.10).

$$\frac{A_t}{A_0} = \frac{t_{equiv}}{D_T} = 10^\wedge \left(\frac{-\int_0^\infty 10^\wedge \left(\frac{T_{(t)} - T_{ref}}{z} \right) dt}{D_{Tref}} \right) \tag{1.14}$$

A non-linear estimation procedure can be used for minimising the sum of squared errors (SSE), employing a quasi-Newton search algorithm, between experimental and predicted residual enzymatic activities, defined in eq. (1.15), where n is the number of experimental runs, subscript 'experimental' indicates experimental data and subscript 'model' indicates prediction from model (eq. 1.14). Different estimate values must be tested to prevent obtaining local minima (MATSUI et al., 2008).

$$SSE = \sum_{i=1}^n \left[\log \left(\frac{A}{A_0} \right)_{i,experimental} - \log \left(\frac{A}{A_0} \right)_{i,model} \right]^2 \tag{1.15}$$

1.6   Final considerations

Challenges in improved the physical properties and stability of beverages, as well as their enzymatic stability still exist. When fruit juices are processed with the ultrasound technology, there are many possible effects that it could produce, as large particles surface erosion (by cavitation collapse in the surrounding liquid) or particle size reduction (due to fission through interparticle collision or the collapse of cavitation bubbles formed on the surface). However, any work has shown how these effects influence the physical properties and stability behaviour through the structure changes at the molecular and microscopic level, as well as their responses throughout the US process time.

On the other hand, the induced cavitation by ultrasound, also the severe rise in temperature and pressure in localized area, results in high shear rates and generating strong micro-streaming, which can contribute for the enzyme inactivation. However, studies show not only enzyme inactivation with the application of US, but also activation and/or oscillations in enzyme activity. Therefore, it is necessary to better

understand the mechanism of reaction of the enzymes against the ultrasonic energy applied and in turn summarize the reported mechanisms involving activation, inactivation and/or activation/inactivation. As for the effect of ultrasound and thermal processing, most studies show processes thermosonicacion (i.e. the thermal process is performed simultaneously with the process of US), in this context it is difficult to differentiate or to know how and how much influence the US, also their effects could be counteracted by increasing the temperature.

Consequently, the present dissertation was focused on describing and explaining how the US effects change the structure and properties and how it could be improving the stability and physical properties as well as the enzyme stability.

1.7   Objectives

The main objective of this work is to evaluate the effect of ultrasound technology in the physical properties and stability of fruit juices and as a processing method to inactivate the peroxidase (POD) enzyme of coconut water, and as a pre-treatment method to enzymatic sensitization and improve subsequent thermal processing.

The specific objectives were:

Evaluate changes produced by ultrasound technology in peach juice: juice microstructure, rheological properties of juice and serum, sedimentation stability, turbidity and colour during storage and mathematical modelling of relevant evaluated properties.

Study the effect of the US processing in the enzymatic activity of coconut water POD, and correlate the US factors that induce enzyme activation, inactivation or oscillations in its activity.

Evaluate and model the enzyme inactivation kinetics of coconut water POD under non-isothermal conditions, using the ultrasound as pre-treatment to sensitize the enzyme before the heat thermal process.

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2 PEACH JUICE PROCESSED BY THE ULTRASOUND TECHNOLOGY: CHANGES IN ITS MICROSTRUCTURE IMPROVE ITS PHYSICAL PROPERTIES AND STABILITY*

Abstract

Ultrasound is a non-conventional processing technology, which can be used not only for food preservation, but also to improve its properties and quality. This study evaluated the physical properties and stability of peach juice processed by the ultrasound technology. The peach juice processed by the ultrasound technology showed changes in its structure, evidenced by the optical microscopy and particle size distribution, involved steps of cell damage and release of intracellular content, particle size reduction, disruption of the whole cells, polysaccharide size reduction and dispersion of constituents. These effects, depending of the processing time, can trigger different mechanisms with a complex behaviour. The interaction among them and the relative importance of each one change during processing, determining the final rheological properties, pulp sedimentation and serum cloudiness (turbidity). The results indicated that the ultrasound technology can be used to improve the physical properties of peach juice, increasing the stability to pulp sedimentation and serum cloudiness, maintaining or increasing the juice consistency, with insignificant colour changes during the storage.

Keywords: Fruit juices; Ultrasound technology; Physical stability; Physical properties

2.1 Introduction

Ultrasound (US) is defined as sound waves having frequency that exceeds the limit of the human ear (~20 kHz). This technology has been used as alternative to conventional food processing. Based on the frequency range, the applications of ultrasound in food analysis, quality control and processing can be divided into low energy (frequencies higher than 100 kHz at intensities below 1 W/cm^2,) and high energy (intensities higher than 1 W/cm^2 at frequencies between 20 and 500 kHz) (AWAD et al., 2012). The use of power ultrasound (high energy) in processing, which due the acoustic and hydrodynamic cavitations are able to induce chemical and physical changes in different food systems (CHEMAT; ZILL E; KHAN, 2011; AWAD et al., 2012). In fluid food systems, such as juices, subjected to sonication, a number of

physical and mechanical effects can result. Large particles of a liquid suspension are subject to surface erosion (by cavitation collapse in the surrounding liquid) or particle size reduction (due to fission through interparticle collision or the collapse of cavitation bubbles formed on the surface) (MASON; PANIWNYK; LORIMER, 1996). Several studies have evaluated the use of the US technology as alternative for fluid food processing facilitating operations such as emulsification (GAIKWAD; PANDIT, 2008), modifying the functional properties of different food proteins (YANJUN et al., 2014) and assisted the extraction of various food and bioactive compounds (BARBA et al., 2015; ROSELLÓ-SOTO et al., 2015).

In fact, many works were carried out relating the processing of fruit juices using the US technology as alternative for total or partial substitution of thermal processing (ZINOVIADOU et al., 2015). Thermosonication with low temperature could enhance the inactivation of enzymes and microorganisms and it was used as a potential preservation technique in pear juice (SAEEDUDDIN et al., 2015), tomato juice (WU et al., 2008; TEREFE et al., 2009; ERTUGAY; BAŞLAR, 2014), watermelon juice (RAWSON et al., 2011), orange juice (WALKLING-RIBEIRO et al., 2009), apple juice (BASLAR; ERTUGAY, 2013; ABID et al., 2014), grapefruit juice (AADIL et al., 2015), among others.

At present, the effect of US technology on the physical properties of food products such as the colour degradation and quality parameters in orange juice (TIWARI et al., 2008, 2009), cloudy quality of apple juice (ERTUGAY; BAŞLAR, 2014), colour stability and apparent viscosity of pineapple juice (COSTA et al., 2013), colour stability, solid deposition and apparent viscosity of cactus pear (CRUZ-CANSINO et al., 2015), colour and sensory quality (appearance, texture, taste and aroma) of soursop juice (DIAS et al., 2015), has been studied. These previous works indicated that this technology could be used to increase and improve the properties of juices such as the consistency (apparent viscosity), colour, cloudy stability and its sensory acceptance.

However, it is still necessary to improve the description of how the US technology promotes desirable physical properties of fruit juices widely consumed nowadays. Further, the mechanisms by which the US technology changes the food properties must be described, as well as correlate the process with the food structure and properties. In fact, it is expected that the US technology could prevent the pulp

sedimentation by disrupting the suspended particles, reducing the need for addition of stabilizers to the juice, which is highly desirable from an industrial point of view.

Therefore, the present work evaluated the effect of the US technology on the physical stability of peach juice, evaluating the changes in its microstructure, particle size distribution (PSD), pulp sedimentation, serum cloudiness, juice colour and rheological properties of the peach juice and peach serum.

2.2 Materials and methods

2.2.1 Peach juice

In order to guarantee standardization and repeatability, a commercial pasteurized peach pulp (DeMarchi, Brazil) was used to obtain the peach juice. From then, the pulp was diluted in distilled water using 50% of pulp, according to the Codex Alimentarius (2005). The characteristics of the prepared juice were pH of 4.36 ± 0.02, 4.05 ± 0.08% of total solids and 3.00 ± 0.00 ºBrix. As established in the Codex Alimentarius (1995) and described in previous work (KUBO et al., 2013), potassium sorbate (1000 mg·kg^{-1}) was added to the peach juice in order to allow microbial stability during the 21-day of storage evaluation at 25 °C.

For the rheological study of the continuous phase (juice serum), the juice prepared as described above was stored for 24 h at 5 °C (Biplex fridge 340 L, Consul, Brazil) in order to guarantee the complete pulp hydration. Then, the serum was obtained by centrifugation of the juice at 3300 g for 10 min at 25 °C (ROTINA 420 R refrigerated centrifuge, Hettich, England).

2.2.2 Ultrasonic processing

The peach juice sample (150 mL) was placed in a jacketed vessel with water circulation in order to control the process temperature (the temperature of all processing times was maintained at 22 ± 3 ºC). The samples were processed at five processing times (0, 3, 6, 10 and 15 min – these conditions were fixed at previous evaluation) using an ultrasonic tip (ECO-SONIC, QR1000 Model, Brazil) with a nominal power of 1000 W, frequency of 20 kHz and a 1.26 cm^2 titanium tip (keeping it at 3 mm

depth in the juice samples – manufacturer recommendations). The ultrasonic intensity and volumetric power of the equipment were 793.65 W/cm^2 and 6.67 W/mL, respectively. Therefore, the energy inputs was 0 kJ/mL for the control sample (0 min of ultrasonic processing), and for the treatments of 3, 6, 10 and 15 min it was 1.20, 2.40, 4.00 and 6.00 kJ/mL, respectively. All the treatments were carried out in triplicate.

2.2.3 Effect of the ultrasound technology on peach juice microstructure, physical properties and stability

The effect of US on the juice properties was evaluated from its microstructure (using both optical microscopy and particle size distribution (PSD) evaluation), pulp sedimentation, serum cloudiness, juice colour, and rheological properties (time-dependent and steady-state shear properties of the juice and serum). The unprocessed sample (coded as US-0min) and the processed samples (coded as US-3min, US-6min, US-10min, US-15min) were compared just after processing and during 21 days of storage at 25 °C (B.O.D. TE391, Tecnal, Brazil), in order to understand the effect of the US on the physical stability of the juice. The analyses were carried out with three replicates.

Optical microstructure

The samples (~20 µL) were deposited and dispersed on a glass slide to be observed under an optical microscope (Olympus system microscopy model BX41, Japan) equipped with a digital colour camera (Q-Color 3 OLYMPUS America INC, including the SQ Capture 2.90.1 Ver. 2.0.6 Software, Canada). The images were captured at least in quintuplicate for each sample using the 10x objective.

Particle Size Distribution (PSD)

The PSD of juice was determined using the Laser Diffraction Particle Size Distribution Analyzer (Partica LA-950V2 Laser Particle Size Analyzer HORIBA, Japan). Data obtained were analysed using the equipment software (HORIBA LA-950 for Windows, Japan).

In addition to the PSD, the volume-based mean diameter (D[4,3] (eq. 2.1), and the area-based mean diameter (D[3,2], (eq. 2.2), were evaluated. Both equivalent

diameters were evaluated since the D[4,3] is highly influenced by large particles whereas D[3,2] is more influenced by the smaller ones (BAYOD et al., 2007; ZHANG et al., 2010; BENGTSSON; TORNBERG, 2011; SCHULTZ; BARRETT; DUNGAN, 2014).

$$D[4,3] = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3} \qquad (2.1)$$

$$D[3,2] = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \qquad (2.2)$$

Pulp sedimentation

Pulp sedimentation was evaluated using 25 mL graduated cylinders filled with the samples and stored at 25 °C (BOD TE391, Tecnal, Brazil) for 21 days (simulating a shelf life evaluation). The evaluation was carried out every day during the first 6 days and further five times during the next 15 days. In addition, during the first day, it was carried out the evaluation of sedimentation in the first 24 h in order to understand the juice sedimentation behaviour during the moment to be consumed. As described by Kubo; Augusto; Cristianini (2013); Leite; Augusto; Cristianini, (2015) and Silva et al. (2010) the sedimentation index (IS in %) was obtained according to eq. (2.3).

$$IS \% = \frac{S_{(t)}}{V} \cdot 100 \qquad (2.3)$$

Serum cloudiness (turbidity)

The serum cloudiness was also evaluated during the storage. For that, aliquots (6 mL) of the stored juice were centrifuged at 3300 g for 10 min at 25 °C (ROTINA 420 R refrigerated centrifuge, Hettich, England). The supernatant (i.e., the juice serum) was poured into 3 mL cuvettes and placed in a UV-visible spectrophotometer (Uvmini-1240, SHIMADZU, Japan) calibrated with distilled water. The absorbance at 660 nm was measured and directly related to the turbidity (LIANG et al., 2006; SILVA et al., 2010; ZHANG et al., 2010; ZHOU et al., 2010; KUBO; AUGUSTO; CRISTIANINI, 2013).

Instrumental colour

The instrumental Colour was measured using a Hunter Lab spectrophotometer (MiniScan® XE Plus, Hunter associates Laboratory Inc., USA), using the D65° illuminant with an angle of observation of 10°. For the analysis, 50 mL of juice sample at 25 °C was used. The samples were placed in glass cuvette, and three readings were obtained for each replicate. The colour was recorded using the CIE (*Commission Internationale d'Eclairage*) colour scale to measure the parameters of L*, a* and b*. Numerical values of L*, a* and b* were used to obtain the colour difference (ΔE, eq. (2.4)) using the control juice (US-0min) as reference.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (2.4)$$

Rheological properties

As the fruit juices are composed by an insoluble phase (the dispersed phase or pulp, which is composed by fruit tissue cells and their fragments, cell walls and insoluble polymer clusters and chains) dispersed in a viscous solution (the serum, which is an aqueous solution of the intercellular components, such as soluble polysaccharides, sugars, salts and acids) (AUGUSTO; IBARZ; CRISTIANINI, 2012a), the rheological evaluation was carried out on both peach juice and peach serum.

For the whole peach juice, the rheological evaluation was carried out using a controlled stress (σ) rheometer (AR2000ex, TA Instruments, USA) and a cross hatched plate-plate geometry (40 mm of diameter) with a gap of 1000 μm (which was determined following the procedure described by Tonon et al. (2009). The sample temperature was maintained constant at 25 °C using a Peltier system, and each sample was analysed in triplicate.

The peach juice samples were firstly placed in the rheometer and maintained at rest for 5 min before shearing. After resting, the samples were sheared at a constant shear rate (300 s^{-1}) for 600 s, while the shear stress was measured for the time-dependent (thixotropic) behaviour evaluation. After the time-dependent shear period, a stepwise linear decreasing protocol (300 s^{-1} to 0.1 s^{-1}) was used to guarantee steady-state shear conditions for the flow behaviour evaluation.

The time-dependent rheological properties were evaluated using the first part of the protocol. The shear stress decay was evaluated using the Figoni and Shoemaker

model ((FIGONI; SHOEMAKER, 1983); eq. (2.5)). The steady-state shear rheological properties were evaluated using the second part of the protocol. After verifying the obtained flow behaviour, the product was modelled using the Herschel-Bulkley model (eq. 2.6).

$$\sigma = \sigma_e + (\sigma_i - \sigma_e). \exp(-k_{FS}.t) \tag{2.5}$$

$$\sigma = \sigma_0 + k. \dot{\gamma}^n \tag{2.6}$$

The serum phase of the peach juice was evaluated using the same rheometer described, with a 60 mm diameter cone-plate geometry instead (angle 2°) at 25 °C. The samples were submitted to a stepwise linear decreasing protocol (300 s^{-1} to 0.1 s^{-1}) to guarantee steady-state shear conditions for the flow behaviour evaluation. Due to the observed pattern, the rheological behaviour was modelled using the Newton equation (eq. 2.7).

$$\sigma = \eta. \dot{\gamma} \tag{2.7}$$

Statistical analysis and mathematical modelling

A completely randomised design (CRD) was conducted. The analysis of variance (ANOVA) was carried out with a significant probability level of 95% ($p < 0.05$); significant differences between mean values were determined by *Tukey's HSD* (Honest Significant Difference) post-hoc pairwise comparison test. Statistical analyses were determined using software IBM SPSS Statistics 21 (IBM SPSS, USA).

When relevant, some of the properties evaluated were modelled as a function of the ultrasonic processing time using non-linear regression and the Software STATISTICA 7 (StatSoft, USA). The rheological behaviour was modelled using non-linear regression with the software CurveExpert Professional (v.1.6.3, http://www.curveexpert.net/, USA) at a significant probability level of 95%.

2.3   Results and Discussion

2.3.1   Optical microstructure

Figure 2.1 shows the microstructures of the unprocessed and ultrasonic processed peach juice samples (it is important to highlight that the nomenclature "unprocessed" is referred to the sample that has not been processed by the ultrasound technology; however, as all the samples were obtained from a pulp with the same previous process – consequently, the differences among them can be attributed to the ultrasound technology, justifying the following discussion). By being juices, all the samples are composed by a serum phase constituted of water and the intracellular soluble material, obtained from the fruit grinding, and a dispersed phase, whose composition changed for each processed sample. Whereas the unprocessed sample (US-0min) still showed remained whole cells (wc) with intact walls, the ultrasonic processed samples shows different changes in the cell structure, as described below.

The main carotenoids identified in the mesocarp (pulp) of peach fruits are, phytoene, β-carotene, β-criptoxanthin, zeaxanthin, luteoxanthin, and 9-Z-violaxanthin. Carotenoids may be located in the plant cell in different plastids such as, etioplasts, amyloplasts, elioplastos, leucoplasts, and principally in chromoplasts (GROSS, 1979; GIL et al., 2002; MELÉNDEZ-MARTÍNEZ; VICARIO; HEREDIA, 2004; CAPRIOLI et al., 2009; ORDÓÑEZ-SANTOS; VÁZQUEZ-RIASCOS, 2010). In fact, plastid groups (pg) containing carotene can be seen in the unprocessed sample in localized areas of the cell.

In the first sonication condition (US-3min), a small cell disruption and the dispersion of the intracellular components (ic) into the cell are observed, in contrast with the initial compartmentation in the unprocessed juice (US-0min). Probably, the first effect produced by the ultrasonic process is the movement and possible rupture of intracellular structures, including plastid membrane rupture, allowing the carotenoid dispersion into the cytoplasm, while the cellular disruption is still low or absent. These effects are probably due to the induced shear stress and cavitation, not only outside the cells (in the juice serum), but also into the intracellular liquid. This is an interesting and novel result, which can have further implications.
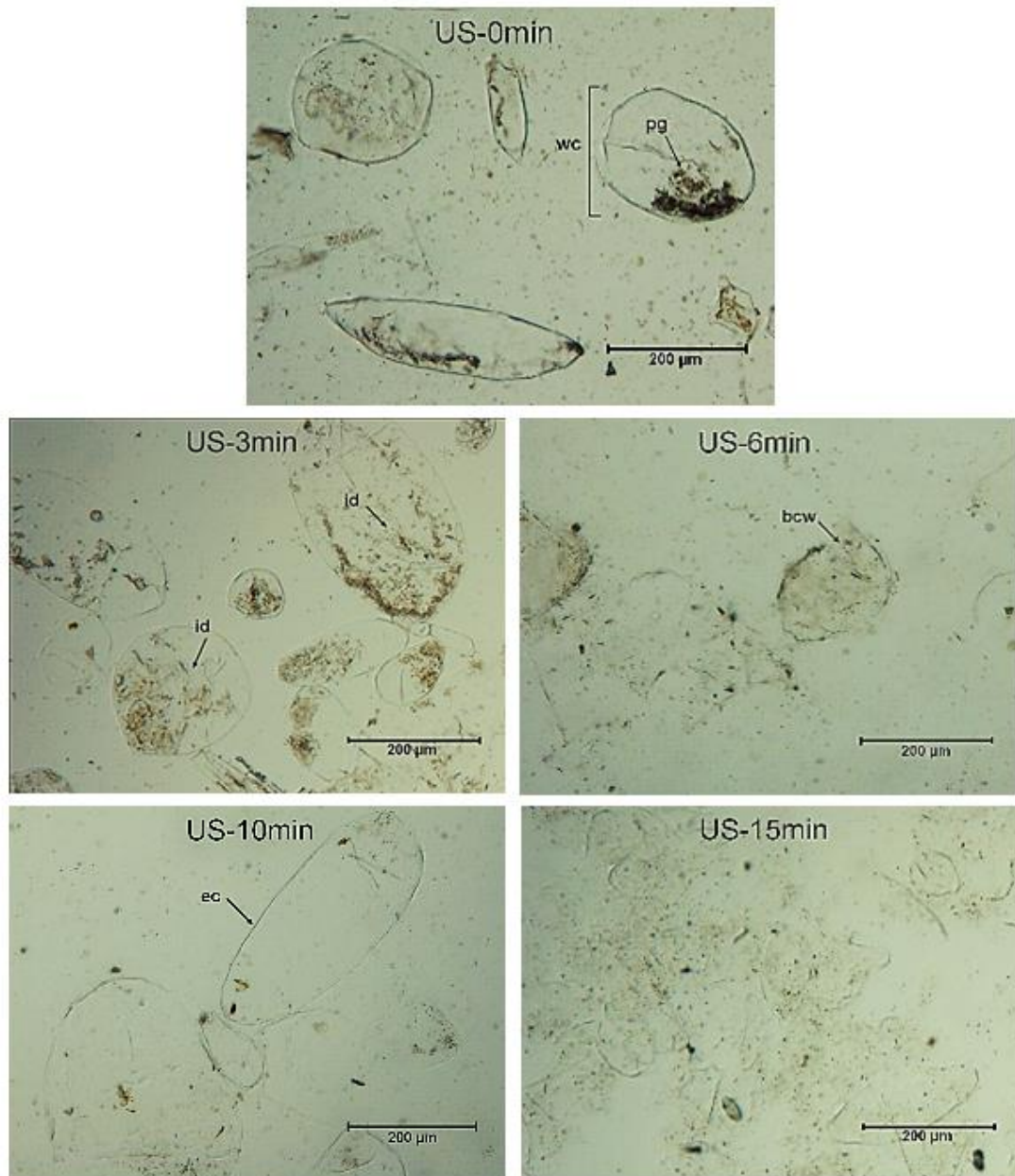
Figure 2.1 – Effect of ultrasonic processing on the peach juice microstructure: optical microscopy (OM) using a 10x objective. Specific regions highlighted as whole cell (wc), plastid group (pg), internal dispersion (id), broken cell wall (bcw) and empty cell (ec). The scale bar shows 200 µm

As expected, by increasing the ultrasonic processing time, more damage in the cellular structure is observed.

After 6 min of processing (US-6min), the cell wall broken (bcw) became more pronounced, allowing the release of intracellular compounds. It continued until 10 min of processing (US-10min), when empty cells (ec) can be observed. Where the perimeter structure of these cells is still conserved, although the ultrasonic processing

results in local break discontinuities in the cell wall and, consequently, cell components release. At higher process time (US-15 min), however, large amounts of small particles composed by internal components and cell wall constituents are dispersed in the juice serum, highlighting the cell disruption, which is observed by many authors, such as Mason et al. (1996). Due to this effect, the US technology facilitated the release of cellular components and different mass transfer unit operations, such as the anthocyanin extraction in grape pomace (BARBA et al., 2015), improving the quality (increased sugars and phenolic compound content and antioxidant activity) and yield in guava juice (NGUYEN; LE; LE, 2012), and slightly affecting the all-trans lycopene concentrations and in vitro bioaccessibility of tomato pulp (ANESE et al., 2015).

Therefore, it was shown that the ultrasound technology can disrupt the peach juice cells, which can lead to both desirable and undesirable consequences. Further, it was demonstrated that this cell disruption takes place following different steps, whose behaviour is more complex than the general description in the literature. Some possible desirable consequences can be an increase in the product consistency, leading to a better sensorial acceptance and reduction of pulp sedimentation, as well as a possible increase on the carotenoid bioaccessibility. On the other hand, a possible undesirable consequence can be the increase on the nutrients and pigment exposure to the oxygen, leading to a reduction on the nutritional content and colour degradation. In fact, the changes in the juice physical properties were studied in the present work, being described in the following sections. Further studies relating to the nutritional aspects are still needed.

2.3.2   Particle Size Distribution (PSD)

Figure 2.2 shows the particle size distribution (PSD) of the unprocessed and the ultrasonic processed peach juices. It can be seen that the PSD of the samples processed for 3-10 min (US-3min, US-6min, US-10min) are similar, and differ slightly from unprocessed sample (US-0min) and processed for 15 min (US-15min). Figure 2.3 shows the mean particle diameter based on the volume ($D[4,3]$ – eq. (2.1)) and area ($D[3,2]$ – eq. (2.2)) for the evaluated samples. It is noteworthy that the peach juice is a polydisperse system (ZHOU et al., 2010), evidenced by the difference between the mean particle diameters $D[4,3]$ (~269.69±6.89 µm) and $D[3,2]$ (~156.14±2.72 µm) of

the unprocessed samples. A reduction in the suspended particles due to the ultrasonic processing is expected, being also observed in diluted avocado puree (BI et al., 2015) and tomato juice (WU et al., 2008).

However, for the volume-based diameter, a significant decrease ($p<0.05$) in relation to the unprocessed sample (US-0min) is shown only by the sample processed for 15 min (US-15min). Further, in this condition, the diameter reduction for the D[4,3] (18% of reduction in relation to the unprocessed sample) was lower than the reduction in the D[3,2] (53% of reduction in relation to the unprocessed sample). Since the D[4,3] is highly influenced by large particles and the D[3,2] is more influenced by the smaller ones (BENGTSSON; TORNBERG, 2011; LOPEZ-SANCHEZ et al., 2011), this result indicates a considerable increase in the number of small particles when the juice is processed for 15 min, in relation to the other conditions.

On the other hand, analysing the process stages, different patterns were observed.

From 0 to 3 min of processing, it can be seen (Figure 2.3) that in the D[3,2], a significant decrease occurred, while there is no significant change on the D[4,3]. Consequently, the changes in the smaller particles (cell fragments and different cell constituents, isolated or as aggregates) seem to be more important than those on the bigger particles (whole cells). It can demonstrate a possible difference in the mechanical resistance between the whole cells and its fragments, which can be interpreted by different possible mechanisms: the different compositions, the region exposed to the impinging jets formed in the serum cavitation (that is the cell wall in the whole cells, and also the internal membranes in the cell fragments), the internal cell turgor/pressure and consequent mechanical resistance and/or the different compositions of the serum and intercellular liquid, leading to different vapour pressure and susceptibility to cavitation. This result highlights once more that the suspended particles disruption by the ultrasound technology is a complex phenomenon, which must be better understood.
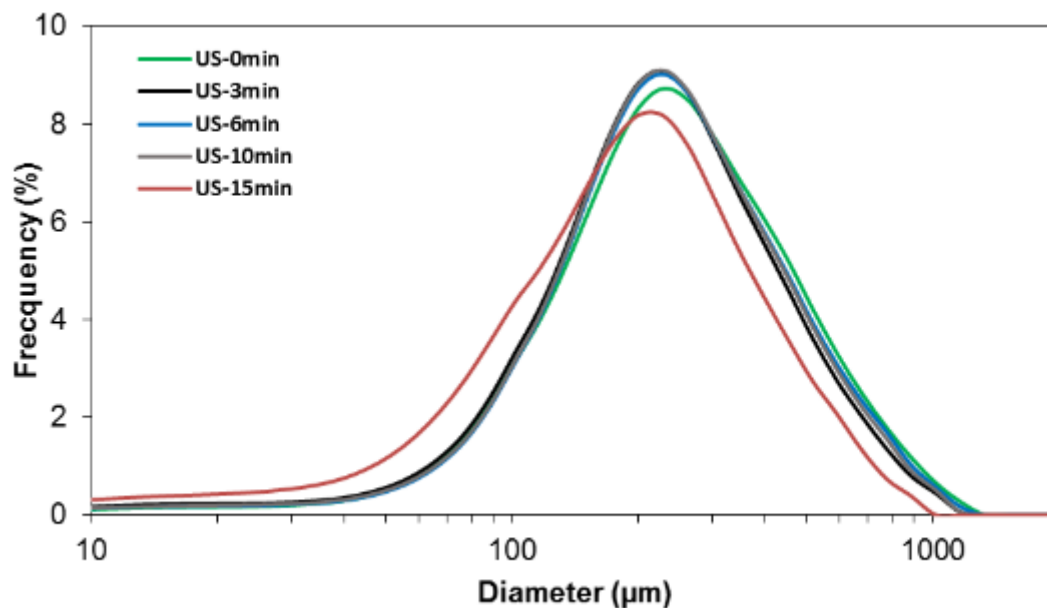
Figure 2.2 – Effect of ultrasonic processing (0–15 min) on particle size distribution (PSD) of the peach juice

From 3 to 6 min of processing, it was observed an increase in D[3,2], whose mean value is maintained from 6 to 10 min of processing and then it is reduced in the 15 min of processing. The observed behaviour may be due to the entry of water into the cell, generating swelling of the cells as a result of the change in the permeability of the cell wall. It can also be due to the presence of aggregates in the samples processed at 6 and 10 min, formed after a progressive reduction of the small particles and a progressive change in the dispersion media composition, due to the intercellular material release to the serum. However, although a slight change in the PSD (Figure 2.2) was observed from 0 to 3 min, only a very small change was observed when the samples were processed at 3, 6 and 10 min. Besides, no statistical changes were observed on D[4,3] from 0 to 10 min, demonstrating that although there was a reduction in the particle size, the large particles were still present. In fact, it is in accordance with the microstructure evaluation (Figure 2.1). Finally, at 15 min of ultrasonic processing, an increase in the distribution due to the reduction in the particle sizes, was observed (Figure 2.2) and a significant decrease in the D[4,3], D[3,2] (Figure 2.3), suggesting that at this time there was a greater number of smaller particles, and decreased the number of large particles.
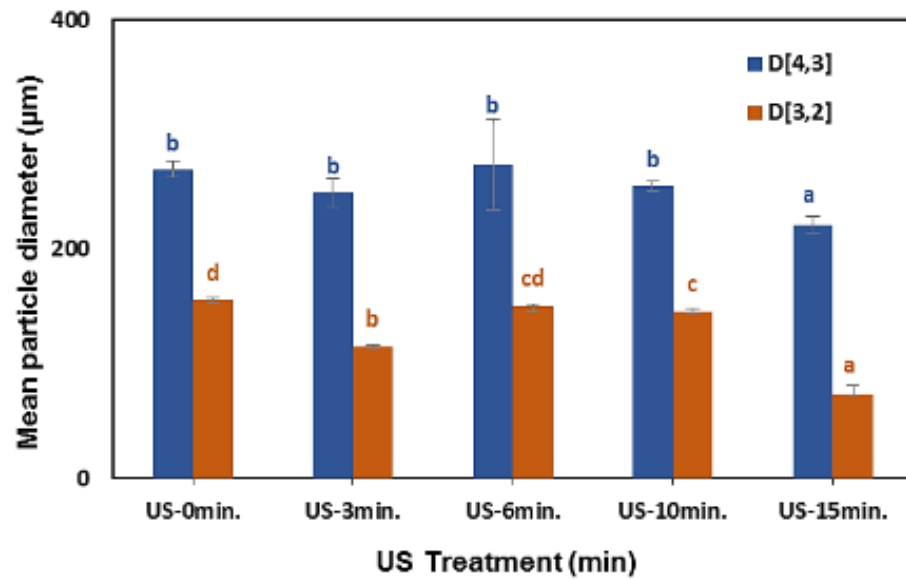
Figure 2.3 – Mean particle diameter for Volume-based mean diameter (D[4,3]) and Area-based mean diameter (D[3,2]). Vertical bars represent the standard deviation in each value; different letters indicate significant differences (p<0.05) for the same parameter

Under these conditions, since the constituents exposed by cell disruption change the properties of the particles and serum juice, and the particle surface area is increased, the particle–particle and particle-serum interactions are improved (AUGUSTO; IBARZ; CRISTIANINI, 2012b; KUBO; AUGUSTO; CRISTIANINI, 2013). The structural changes obtained after the ultrasonic processing result in different mechanisms with an important impact in the pulp sedimentation, turbidity and rheological behaviour of the juice. Further discussion is provided in the "general discussion" section.

2.3.3 Pulp sedimentation

Figure 2.4 shows the sedimentation index (IS) of the peach juice submitted to the different treatments. Both the control sample (US-0 min) and the US processed sample for 3 min (US-3 min) showed an increase of pulp sedimentation with time of storage. Even so, the US-3 min sample shows a considerable pulp sedimentation reduction in relation to the unprocessed juice. Further, there was not any sedimentation in the US processed samples after 6, 10 and 15 min of processing. This result

highlights a possible industrial application of the ultrasound technology in food processing.
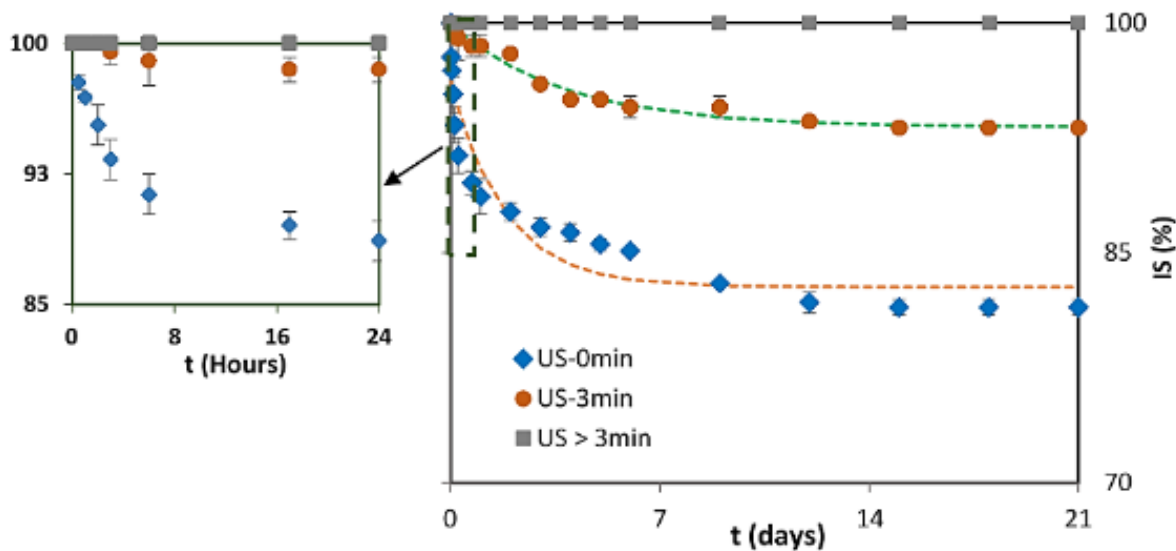
Figure 2.4 – Effect of the ultrasonic processing on peach juice pulp sedimentation. The dots are the mean values, the vertical bars are the standard deviation and the dashed curves are the models described in Table 2.1. As there was no sedimentation in the samples after 6, 10 and 15 minutes of processing, they are symbolized together as "US > 3min". In the detail, the IS (%) during the first 24 hours

The juices are complex systems, which can be considered a dispersion containing pulp particles (dispersed phase) colloidally dissolved in an aqueous solution (or serum - continuous phase), constituted by both macromolecules (as pectins) and low-molecular weight components (sugars, organic acid, salts, etc.) (GENOVESE; LOZANO, 2006; MOLLOV et al., 2006) from the intercellular material. The suspended particles are pieces of tissues, intact and disrupted cells and its fragments, whose walls are insoluble (AUGUSTO; IBARZ; CRISTIANINI, 2012a). The intact cells in the peach juice here evaluated were in the order of 200-300 µm x 100-300 µm (Figure 2.1). The changes in the juice microstructure can describe the observed behaviour in relation to the pulp sedimentation.

The stability of large particles (>10 µm) suspended in a fluid is governed by the hydrodynamic forces (GENOVESE; LOZANO; RAO, 2007), and the sedimentation mechanism can be mainly explained by the Stokes Law. According to the Stokes Law, for spherical and rigid particles, without interactions, dispersed in a Newtonian fluid,

the particle sedimentation velocity is directly proportional to its squared diameter, the acceleration imposed and the difference between the particle and dispersant medium density, being inversely proportional to the dispersion medium viscosity. Thus, the predominant large particles in the unprocessed sample (i.e., the US-0min) sediment faster than the processed sample, which can be seen right in the first 24 h, where the pulp sedimentation reached 61% of the total sedimentation at the equilibrium (which took ~15 days; after that the IS was constant). Further, the cell damage and disruption, with the consequent release of intracellular compounds, increased the serum viscosity, which can also explain the reduction in the particle sedimentation.

On the other hand, the reduction on the particle size can result in a colloidal system, also increasing the relative particle surface area and possible interactions (BAYOD; TORNBERG, 2011). Therefore, even if the inter-particle interaction was low, the overall interaction was increased with the particle size reduction and increased in the amount of small particles. Moreover, the disruption of cell releases intracellular material, which changes not only the serum viscosity, but also its interaction properties with the particles.

Further, the stability of colloidal aggregates depends on the balance between attractive van der Waals forces and repulsive electrostatic forces (GENOVESE; LOZANO, 2006; GENOVESE; LOZANO; RAO, 2007). If the attractive forces increase more than the repulsive ones, it can result in the formation of aggregates, increasing the pulp sedimentation. In fact, it was not the case of the peach juice, whose ultrasonic processing promoted the juice stability.

It is important to highlight that this result is very interesting by an industrial point of view. By reducing the pulp sedimentation, the ultrasound technology reduces the need for adding hydrocolloids to the juice, which is desirable for many reasons, such as meeting the consumer demand (to reduce the use of additives), simplifying the process (as the hydrocolloid dissolution is a complex step during processing) and reducing the number of ingredients (with both logistic and economic gains).

In fact, different technologies have been studied and applied in order to improve the stability of juices and beverages without the need for ingredients, such as the high pressure homogenization (AUGUSTO; IBARZ; CRISTIANINI, 2012b; MOON; YOON, 2015), the use of enzymes (VENDRÚSCULO; QUADRI, 2008) and the pulsed electric fields (SCHILLING et al., 2008; ALTUNTAS et al., 2011). As explained, the ultrasonic

processing could be an important technology applied in order to prevent juice sedimentation.

Table 2.1 – Mathematical modelling of pulp sedimentation during 21 days of storage (25 °C): Control sample (US-0min) and US-3min samples (IS in % and t in days; mean values ± standard deviation)

Model	$IS = IS_{equilibrium} + \left(IS_{initial} - IS_{equilibrium}\right).e^{-k.t}$	
Treatment	US-0min	US-3min
$IS_{equilibrium}(\%)$	83.3±1.4	93.2±0.3
$IS_{initial}\ (\%)$	97.2±0.9	99.9±0.4
$k\ (1/days)$	1.38±1.19	0.26±0.00
R^2	>0.92	>0.98

Finally, the IS of the US-0min and US-3min was then modelled as a function of storage time (at 25 °C) using an exponential decay function, with good coefficient of determination (R^2>0.92). The model and parameters are shown in Table 2.1. It is interesting to observe that the evaluated peach juice showed a pulp precipitation behaviour faster and more pronounced than other fruit juices, such as the tomato juice. While the IS at the equilibrium was ~83% and the kinetic parameter was ~1.4 day^{-1} for the evaluated peach juice, these values were ~70% and 0.07 day^{-1} for tomato juice (KUBO; AUGUSTO; CRISTIANINI, 2013). Even so, the ultrasound technology has been proven efficient to avoid this quality fault on peach juice.

2.3.4 Serum cloudiness (turbidity)

Figure 2.5(A) shows the serum cloudiness (turbidity) just after the ultrasonic process, where significant variations and a complex behaviour in relation to the ultrasonic processing were observed. The unprocessed sample (US-0min) was less cloudy than the treated samples only just after the process; further, the turbidity firstly increased and then decreased along the US treatment. Figure 2.5(B) shows the serum cloudiness for all samples with different treatments along 21 days of storage, where

only a slight variation during storage can be observed, possibly due to particle aggregation.
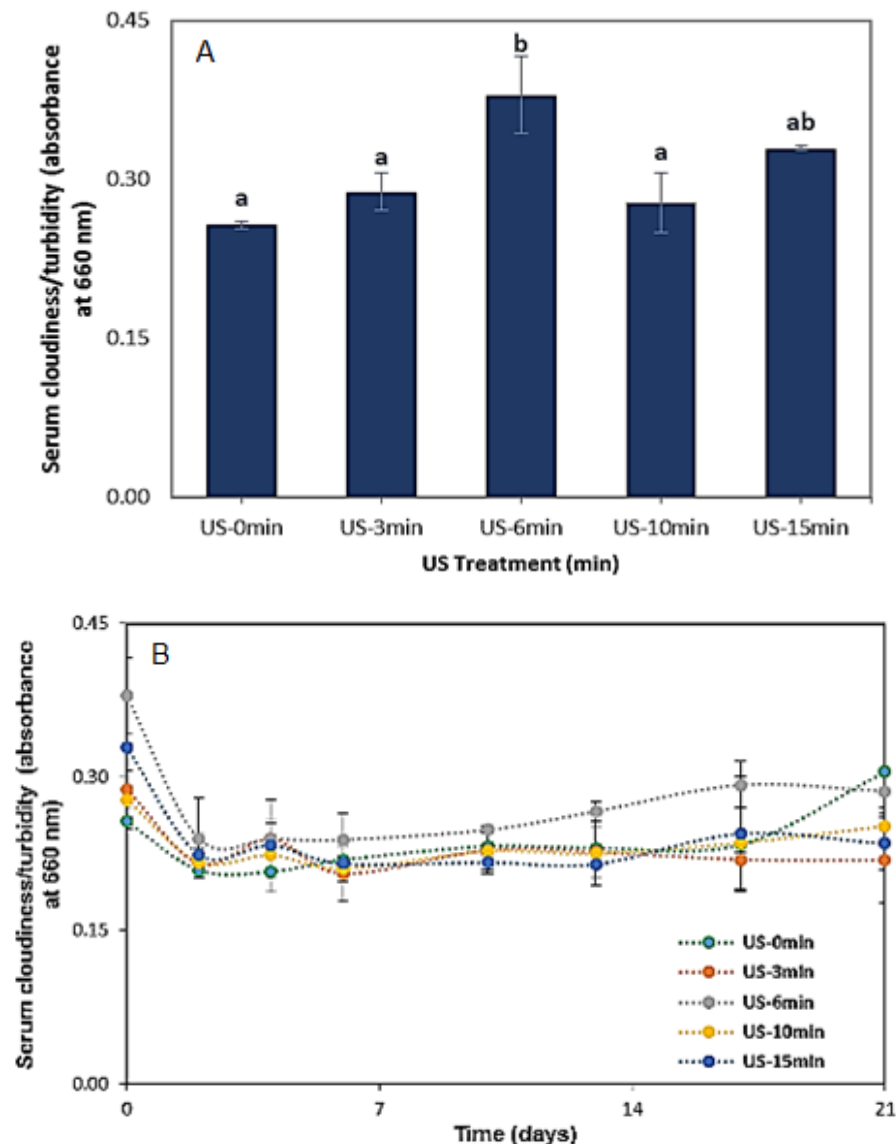
Figure 2.5 – Effect of the ultrasonic processing on the peach juice serum cloudiness just after process (A). Different letters indicate significant differences among treatments ($p < 0.05$). In addition, the peach juice serum cloudiness throughout 21 days of storage at 25 °C (B). The curves are only to facilitate the interpretation. Vertical bars are the standard deviation for each value

It is important to notice the meaning of increase or decrease the serum cloudiness, correlating it with the juice structure and processing, but also describing the way as this evaluation is conducted. During this evaluation, the juice sample is centrifuged (at the same condition of acceleration for all the samples), and only the

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serum (supernatant) is evaluated in the spectrophotometer in relation to its absorbance. The absorbance is thus directly related to the sample cloudiness/turbidity, being the suspended particles responsible for the absorption of radiation. Due to the Stokes Law, the larger particles are easily precipitated and the smaller particles tend to remain in suspension after centrifugation. The particles that remain in suspension increase the absorbance values and the sample turbidity, as observed in Figure 2.5(A). However, when the particle size is reduced, the importance of the non-hydrodynamic forces (electrostatic, Van der Waals) is increased due to the increase of the particle surface area. As a result, aggregates can be formed, whose behaviour during centrifugation and the turbidity analysis is more complex (KUBO; AUGUSTO; CRISTIANINI, 2013). Therefore, small particles can form aggregates, which will sediment during the centrifugation, reducing the serum absorbance. In fact, it explains the first increase and then decrease on the peach juice serum cloudiness in relation to the ultrasonic processing time (Figure 2.5(A)).

Similar results were reported by Cruz-Cansino et al. (2015), during the US treatment of cactus pear juice. The authors obtained a high amount of fine particles in the supernatant after centrifugation of the processed samples. On the other hand, Zhou et al. (2010), obtained peach juice particles more uniform and a higher turbidity when processed by the high-pressure carbon dioxide (HPCD) than the unprocessed juices. In contrast, Silva et al. (2010) processed pineapple juice by the high pressure homogenization technology, reducing the particle size and serum cloudiness with increasing homogenisation pressure.

2.3.5 Instrumental colour

Figure 2.6 shows the parameters of L* (lightness), a* (redness: green to red), b* (yellowness: blue to yellow), and ΔE (total colour change, eq. (2.4)) as a function of the ultrasonic processing and throughout the 21 days of storage at 25 °C. It can be seen that the ultrasonic processing of peach juice resulted in small changes on all the colour parameters, both just after processing and during storage.

The value for L* increased with ultrasound treatments longer than 3 min, indicating that the higher the ultrasound treatment, the samples are clearer; while during the storage no significant variations in L* value were observed. With regard to

the value of a* and b*, no significant changes were observed among the US processed and unprocessed samples. However, for all samples a very slight saturation to the green and decrease of the yellow colour were observed during the storage time. It can be explained by the increase in the light reflection due to small particles (with high area and amount) and a possible degradation of peach pigments during storage (due to any dissolved oxygen).
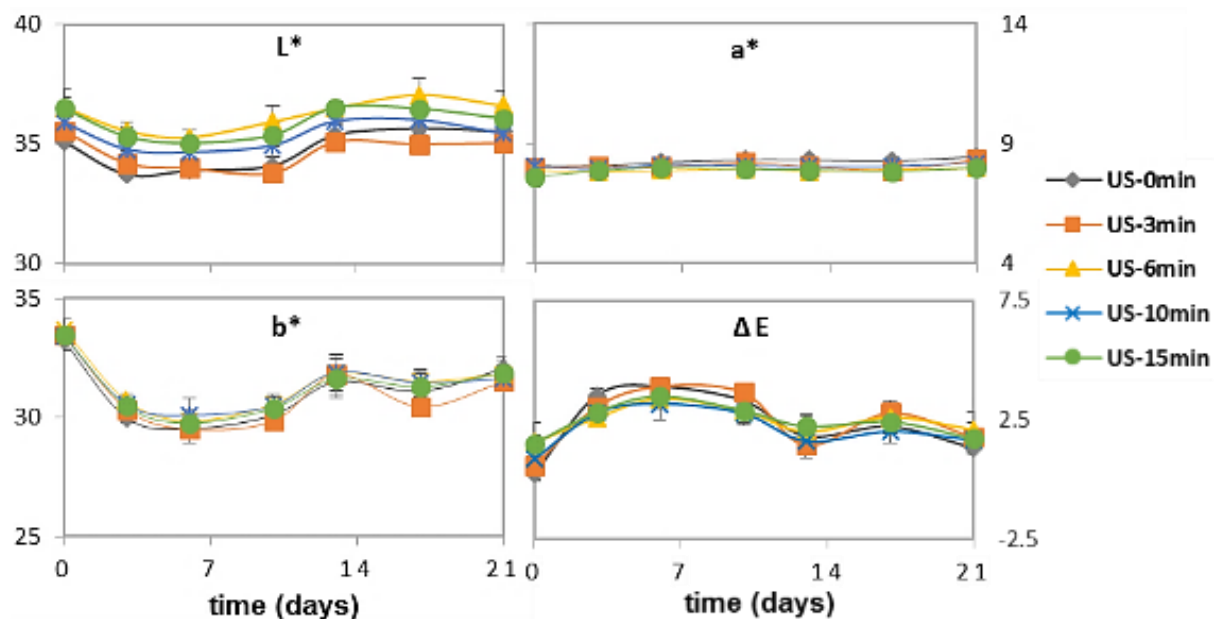
Figure 2.6 – Effect of the ultrasonic processing on the colour of peach juice throughout 21 days of storage at 25 °C. Vertical bars are the standard deviation for each value. The curves are only to facilitate the interpretation

Similar results were observed in other juices processed by the ultrasound technology isolated or in combination with mild temperatures, such as the apple juice (ABID et al., 2014), orange juice (TIWARI et al., 2008) and watermelon juice (RAWSON et al., 2011). However, no significant differences were observed in L*, a* and b* values after the ultrasonic processing (750 W/cm^2, 50% power, 20 kHz, 1–10 min) of diluted avocado puree (BI et al., 2015). It is expected that each fruit tissue has its own behaviour in relation to the ultrasonic processing, concerning the susceptibility to cell disruption, intercellular material release and pigment stability. Even so, the increase in lightness value was attributed to different mechanisms in other juices, such

as the partial precipitation of unstable suspended particles and degradation due to oxidative reactions (RAWSON et al., 2011).

Whereas, the total colour change of peach juice (ΔE using the control juice (US-0min) as reference) among evaluated samples showed no significant differences and during storage slight variations were observed (Figure 2.6). Therefore, under the study conditions it was not detectable to the naked eye. Similar result was obtained for guava juice (CHENG et al., 2007).

2.3.6 Rheological properties

2.3.6.1 Effects of ultrasonic processing on the steady-state shear (flow) properties of peach juice

Figure 2.7(A) shows the flow behaviour of the peach juice processed by the ultrasound technology. All the samples showed the shear-thinning with yield stress behaviour. Therefore, the Herschel-Bulkley model was used and its parameters are shown on Table 2.2.

The effect of the ultrasound process on the peach juice rheology showed a complex behaviour, firstly increasing, then decreasing and finally increasing the consistency (i.e., higher values of shear stress (σ) at the correspondent shear rate ($\dot{\gamma}$) - Figure 2.7(A)), in relation to the processing time. This is an interesting and novel result, which can be related with the changes in the peach juice structure induced by the ultrasonic processing.

The juice showed shear thinning behaviour with yield stress, and could be well described by the Herschel-Bulkley model (eq. (2.6)). Table 2.2 shows the effect of the ultrasound process on the Herschel-Bulkley model parameters for the peach juice at 25 °C. Although the consistency index (k) decreased with the increasing of the process time, while the flow behaviour index (n) and the yield stress (σ_0) showed opposite complex behaviour, only the last parameter showed statistical difference. It demonstrates that, although the ultrasound technology can disrupt the suspended peach cells, highly affecting some properties such as the particle sedimentation (section 3.3), it does not considerably change the rheological properties. In fact, it can be interesting in a commercial point of view. Supposing that the current commercial juice shows desirable rheological properties, the ultrasonic processing can avoid the

undesirable pulp sedimentation without impacting significantly the consumer sensorial perception.

Table 2.2 – Effect of the ultrasound technology on peach juice flow properties: Parameters of the Hershel-Buckley model according to the process time (mean value ± standard deviation)*

Process time (min)	σ_0 (Pa)			k (Pa.s^n)			n (-)		
0	0.512	±	0.050 a	0.054	±	0.009 a	0.775	±	0.031 a
3	1.473	±	0.094 bc	0.040	±	0.004 a	0.815	±	0.015 a
6	1.060	±	0.210 b	0.041	±	0.018 a	0.814	±	0.074 a
10	1.606	±	0.102 bc	0.043	±	0.014 a	0.821	±	0.036 a
15	1.795	±	0.182 bc	0.038	±	0.012 a	0.838	±	0.046 a

*different letters indicate significant differences among treatments

The overall result is that the apparent viscosity increased, decreased and then increased with the process time, implying that the changes in the yield stress were the most pronounced effect on the product rheology. As the ultrasound process time increased from 0 min to 15 min, the consistency index (k) was reduced from 0.054 to 0.038 Pa.s^n, or dropped to 70% of the original value. On the other hand, the flow behaviour index (n) increased from 0.775 to 0.838, and the yield stress (σ_0) showed an increase from 0.512 to 1.795 Pa in the same range process time. However, probably due to the high standard deviation values, only the changes in the yield stress (σ_0) were statistically valid.

The observed complex behaviour on the rheology of peach juice processed by the ultrasound technology was not reported in the literature, and it can be related with the changes in the product structure, as described below. We cannot know if the peach juice structure has some particularity that allows this behaviour, if this behaviour was observed only in the present work due to the chosen conditions (i.e., if we had used conditions more severe, probably only the increase on juice consistency would be

observed) or if the other works do not describe this behaviour, although it may have happened. However, each food matrix may respond in a different and unique way, which also can explain this novel result. It highlights the need for further works relating the ultrasonic processing of fruit juices and its effects on the product structure and properties.

For example, a 60% increase in apparent viscosity was observed on tomato paste processed by the ultrasound technology when compared with the same product thermally processed (VERCET et al., 2002). Further, Dias et al. (2015) verified that, in the sensory evaluation of ultrasonic processed soursop juice, the 'texture' parameter presented higher values if compared with control samples (i.e., not processed), which can be related with an increase in the product consistency.

As, previously stated, each material has its own nature, it is expected that the effects of the ultrasound technology can be different. As stated by Soria and Villamiel (2010), the consistency of a food product can be changed permanently or temporarily, either increasing or decreasing the consistency, depending on the ultrasonic intensity.

In the literature there are not many studies that evaluated the rheology of fruit products processed by ultrasound. However, other emerging technology that decreases the particle size (and its distribution) of several food products including fluid foods, is the high pressure homogenization (HPH). Although the main physical mechanism is different between the ultrasonic technology and high pressure homogenization, the final result on the particle size can be used to trace a parallel between the technologies, and consequently helping to understand the rheology changes caused by the reduction on the particle size. For example, using HPH on tomato juice, Augusto et al. (2012b), observed a decrease in the mean particle size and particle size distribution, leading to an increasing in the apparent viscosity. The main reason is that the small particles showed a higher total surface area, leading to a higher interaction among particles, and, consequently, increasing the yield stress and apparent viscosity. Comparatively, Vercet et al. (2002) observed an increase in the apparent viscosity using the ultrasound technology for processing tomato paste. Then, the effects can be attributed to the chance in the particle size caused by cavitation during the ultrasound process.

Further, the tomato juice presented a shear thinning behaviour with yield stress, and after the particle size reduction it presented a higher consistency (AUGUSTO; IBARZ; CRISTIANINI, 2012b). On the other hand, the cashew apple juice also showed

a shear thinning behaviour with yield stress, however a decrease in the consistency was observed after a reduction in particle size (LEITE; AUGUSTO; CRISTIANINI, 2015).

Further, the juice rheological behaviour cannot be described only by the suspended particle changes, being also necessary to evaluate the serum phase.

To enhance the comprehension of the effects, the Peclet Number (Pe; eq. (2.8)) can be used. The Pe is a dimensionless relation between particle transport due to shearing (non-Brownian systems) and diffusion (Brownian systems) (FISCHER et al., 2009).

$$Pe = \frac{\eta_{continuous\_phase} \cdot \overline{r}_{particle}^{3} \cdot \gamma}{k_B \cdot T}$$

(2.8)

Therefore, the rheological characterization ($\eta_{continuous\_phase}$) of the serum phase was conducted for the samples unprocessed (US-0min) and that one processed using US for 15 min (US-15min).

Figure 2.7(B) shows the flow behaviour of the peach juice serum phase, which, as expected, showed Newtonian behaviour (eq. 2.7). Table 2.3 shows the mean viscosity (η) values as a function of ultrasound process time.

Table 2.3 – Effect of the ultrasound technology on the peach juice serum phase viscosity (mean value ± standard deviation)*

Process time (min)	η (Pa.s)		
0	0.00317	±	0.00007 a
15	0.00245	±	0.00002 b

*different letters indicate significant differences among treatments.

The ultrasound process reduced ~23% of the fluid viscosity. This is expected as the ultrasound energy can reduce the molecular weight of polysaccharides, then reducing the viscosity (SORIA; VILLAMIEL, 2010).

For example, it was observed by Tiwari et al. (2010) with solutions of guar gum (1%), xatham gum (1%) and pectin (2%); Seshadri et al. (2003) with high methoxyl pectin solutions; Huang et al. (2007) with corn starch and Baxter et al. (2005) with chitosan.
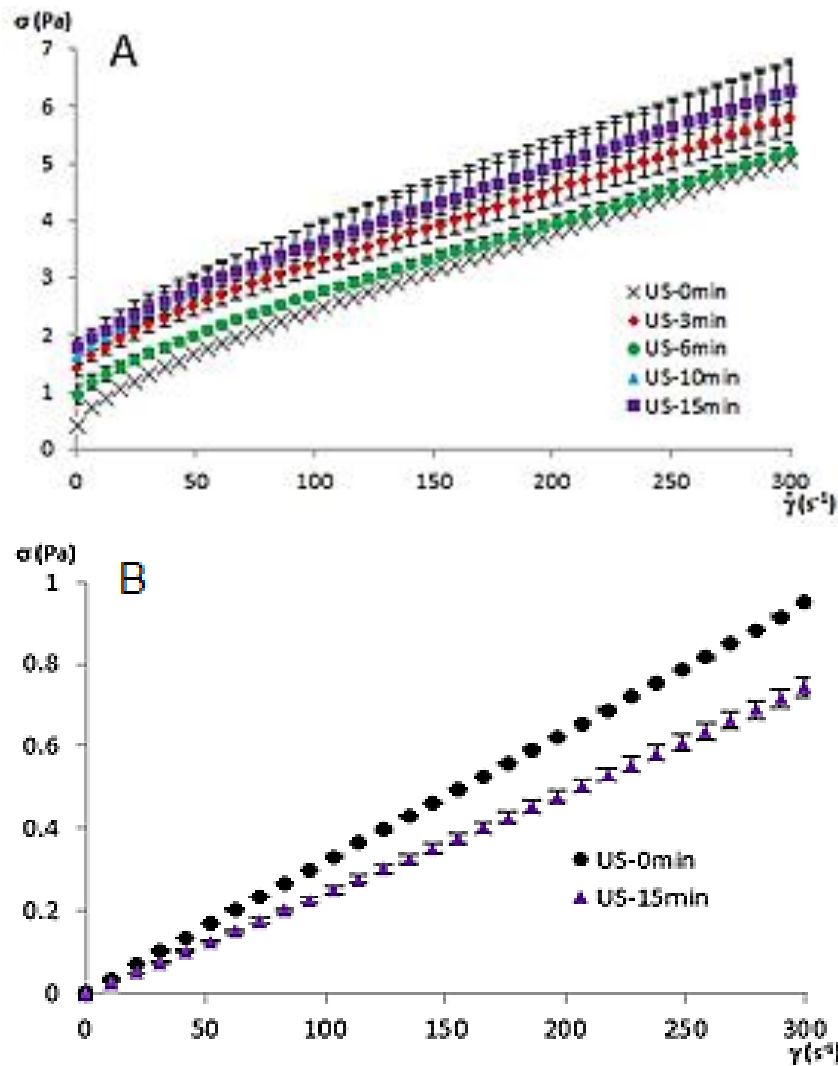
Figure 2.7 – Flow curve at 25 °C for peach juice (A) and peach juice serum phase (B) processed by the ultrasound technology (dots are the mean value; vertical bars are the standard deviation)

Consequently, the ultrasonic processing reduced both the particle size ($\bar{r}_{particle}$; Figures 2.2 and 2.3) and the viscosity of the peach juice serum phase ($\eta_{continuous\_phase}$; Figure 2.7(B)), also reducing the Pe of the juice and approximating the system to the Brownian domain.

The Brownian motion dominates when Pe values are small (in small shear rates and small particles); on the other hand, at higher Pe values (higher shear rates and

larger particles), the shear flow promotes pronounced structural distortions, and the Brownian motion cannot restore the structure of the suspension to its equilibrium state, being then the hydrodynamic forces that dominates, when the shear thinning behaviour occurs (FISCHER et al., 2009). This can also explains the fact of just the yield stress showed statically difference in the present work, since at small Pe the interparticle forces were more relevant, while during high shear, the hydrodynamic forces compensate those differences and all fluid (obtained by the different process conditions) behaves in a similar way.

2.3.6.2 Effects of ultrasonic processing on the time-dependent properties

The effect of ultrasound on the rheological time-dependent properties of peach juice is presented on Figure 2.8. The control sample showed no time dependency (or, at least, a negligible one). However, the processed samples showed thixotropy, which showed a similar complex behaviour stated before, but finally increasing with the ultrasonic processing time. The results were modelled using the Figoni-Shoemaker Model, whose parameters are shown in Table 2.4. The thixotropy is related with the structure change during shear and also the destruction of the internal structure or disaggregation of aggregates due to flow (RAMOS; IBARZ, 1998; CEPEDA; VILLARÁN; IBARZ, 1999; BAYOD; TORNBERG, 2011).

The ultrasonic processing increased the thixotropy of peach juice by increasing the initial shear stress, the equilibrium stress and the difference between the initial and equilibrium stress (Figure 2.8 and Table 2.4). Note that the control sample did not show any time dependent properties, so the initial and equilibrium stresses were the same, without any kinect parameter (k_{FS}) i.e, equal to zero. The sample processed after 15 min showed an initial value for stress (σ_i) of 8.37 Pa, reaching equilibrium (σ_e) at 6.41 Pa, which is 66% and 27% higher than the value of the unprocessed sample.

The ultrasonic processing time impacted more the initial stress rather than the equilibrium stress. It indicates that this technology affects more the forces that are important at low shear (small Pe number) than those important at high shear (high Pe number), being in agreement with the increase in the yield stress.
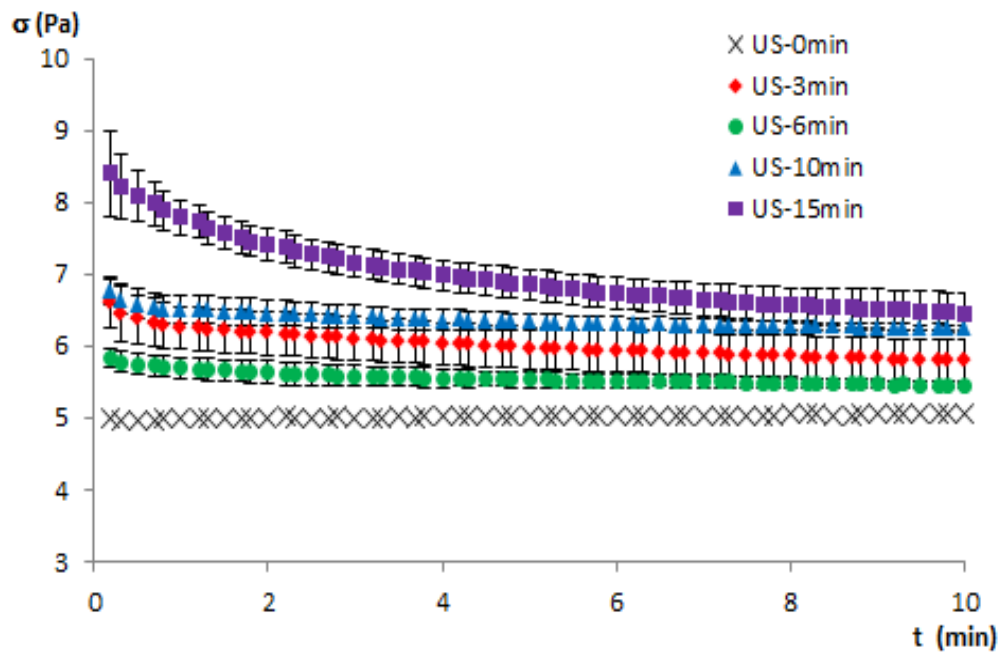
Figure 2.8 – Shear stress with time (thixogram, 300 s^{-1}, 25°C) of peach juice processed by the ultrasound technology (dots are the mean value; vertical bars are the standard deviation)

Table 2.4 – Effect of the ultrasound technology on the peach juice time-dependent rheological properties: Parameters of the Figoni-Shoemaker model according to the process time (mean value ± standard deviation)*

Process time (min)	σ_i (Pa)	σ_e (Pa)	$\sigma_i - \sigma_e$ (Pa)	k_{FS} (s-1)
0	5.03 ± 0.10 a	5.03 ± 0.10 a	0.00	-
3	6.47 ± 0.35 b	5.74 ± 0.30 b	0.73	0.0039 ± 0.0012 a
6	5.90 ± 0.12 ab	5.48 ± 0.18 ab	0.42	0.0030 ± 0.0013 a
10	6.70 ±0.14 b	6.23 ± 0.02 c	0.47	0.0035 ± 0.0012 a
15	8.37 ± 0.50 c	6.41 ± 0.21 c	1.96	0.0051 ± 0.0013 a

*different letters indicate significant differences among treatments

Any other study, in the literature, has evaluated the thixotropy changes on fruit juice processed by ultrasound technology. Once more, another technology that reduces the particles size can be used to explain the obtained results. However, similarly as explained before, different fruit juices showed different behaviours when processed by the high pressure homogenization processing. While the frozen

concentrated orange juice became less thixotropic after a particle size reduction (LEITE; AUGUSTO; CRISTIANINI, 2014), the tomato juice becomes more thixotropic after particle reduction (AUGUSTO; IBARZ; CRISTIANINI, 2012b). It highlights the need for future studies correlating the effect of different technologies on the physical properties of fruit juices.

2.3.7 General discussion: correlation among process-structure-properties and its relevance

The structural changes produced by the ultrasound technology on the peach juice, which are evidenced by the optical microscopy (section 2.3.1) and the Particle Size Distribution (section 2.3.2), involve cell damage, cell disruption, release of intracellular content, particle size reduction, molecular reduction/cleavage and dispersion of constituents, with consequent changes in the interaction forces among particles and between particles and serum. These results can trigger different mechanisms, related with the changes in the properties of the dispersed phase and continuous phase, resulting in the observed complex behaviours.

- The ultrasound technology can affect a liquid dispersion (such as fruit juice) by different mechanisms, which as general mode are listed below. Each mechanism is here described isolated; however, they each one can occur isolated or together during processing, or even so, do not occur, and the relative importance of each one changes during the entire process. In fact, it is function not only of the process conditions, but also to the food structure. Therefore, in general:
- The increase in the serum viscosity and/or the product consistency decreases the rate of pulp sedimentation.
- The reduction on the suspended particle diameter increases the surface area and, consequently, the interaction forces among them and with the continuous phase, increasing the product consistency.
- The reduction on the polysaccharide molecular size decreases the serum viscosity.

- The rupture of the cell wall with the consequent release of intracellular compounds, as electrolytes, increases the serum viscosity and turbidity, increasing the product consistency.

- If the particle size is reduced but large particles are still retained, it increases the dispersibility, consequently the small particles occupy spaces between the large particles, resulting in a "lubricant effect", which decreases the product consistency,

Therefore, the balance among these different mechanisms will determine the final properties related to rheology, pulp sedimentation and turbidity. As noted in the previous sections, each of the evaluated properties does not maintain a constant pattern with the increasing in the ultrasonic processing time, but shows a complex behaviour of increasing/decreasing/increasing (or the opposite). Therefore, the process will be analysed at each step (each processing time), proposing the mechanisms by how the observed complex behaviours took place.

Changes between 0 and 3 min of ultrasonic processing:

According to the microscopy images, during the first condition of processing, although the disruption of remained whole cells still does not occur (or at least occur in a small amount – Figure 2.1), the ultrasonic processing already promotes the reduction of the most sensible fragmented cells or particles that were initially aggregate. Therefore, the small particle amount is increased (which is described by the decrease in the D[3,2]), without changing the amount of large particles (as the D[4,3] does not change) (Figure 2.3). Consequently, the area of the dispersed phase (suspended particles), the interactions (particles-particles and particles-serum), and the particle volume fraction (by reducing the space available for the flow of the continuous phase - serum) are increased, resulting in a lower sedimentation rate (% IS, Figure 2.4), an increase in the product consistency (apparent viscosity) and an increase in the product yield stress (Figure 2.7(A)). Therefore, the observed behaviour in the first condition of ultrasonic processing is the expected one.

Changes between 3 and 6 min of ultrasonic processing:

After 6 min of processing, in the second condition evaluated, according to the optical microstructure (Figure 2.1), the cell wall rupture is higher, releasing intracellular

components to the serum. Whereas, the particle size remains almost the same (Figure 2.2 and 2.3), there are no visible changes in the PSD (Figure 2.2), also statistically the D[3,2] value increases and the D[4,3] value does not change, reflecting two possibilities, the size increases due to the influx water into the cell, that cause swelling of cell and another possibility is the formation of particle aggregates. Even so, there is still a reduction on the suspended particles and increase in the amount of small particles. Consequently, the very fine particles are submitted to colloidal interactions; therefore, they still remain suspended in the serum even after centrifugation, increasing the turbidity (Figure 2.5(A)). On the other hand, a decrease of the product consistency is observed, which can be due to the small particles producing a lubricating effect among the suspended particles. Further, as the ultrasonic process can reduce the polysaccharide molecular size, the serum viscosity could also be reduced, also contributing to the reduction in the overall consistency – in fact, although the serum viscosity was evaluated in this work only after 15 min of processing (Figure 2.7(B)), it is known that the ultrasonic processing promotes a progressive reduction in the serum viscosity (SESHADRI et al., 2003), justifying this discussion. Summarizing, three mechanisms compete to dictate the peach juice overall consistency: the serum viscosity reduction due to the polysaccharide degradation (which would reduce the product consistency), the juice consistency reduction due to the lubricating effect of small particles and the increasing due to the reduction on particle size (increasing the particle volume fraction and interactions). As the observed behaviour was the reduction on the juice consistency (Figure 2.7(A)), apparently the last mechanism is less important in this scenario, while it is difficult to establish which of the first two is the dominant. Therefore, the observed behaviour in the second condition of ultrasonic processing is more complex than the expected one, being described for the first time.

Changes between 6 and 10 min of ultrasonic processing:

As shown in the microscopy evaluation (Figure 2.1), since the release of intracellular compounds is progressing through the partial rupture of the cell wall, the cells apparently are "empty", while retains the frame of its wall. Therefore the same values of D[4,3] and D[3,2] (Figure 2.3), as well as the PSD (Figure 2.2) are observed. However, there is an increase in the product consistency (Figure 2.7(A)) and a decrease in the serum turbidity (Figure 2.5(A)). Even so, it is also expected that there is still a reduction on the suspended particles and increase in the amount of small

particles, which probably form aggregates precipitated during centrifugation (decreasing the turbidity - Figure 2.5(A)). Further, the intracellular material release changes the serum composition and concentration, increasing its viscosity. It can also increase the volume fraction due to the intracellular particles released, causing an effective concentration in the number of particles in the continuous phase. These mechanisms can explain the observed increase in the peach juice consistency (Figure 2.7(A)) and yield stress (Table 2.2) at 10 min of processing. Therefore, although the polysaccharide degradation (which would reduce both the serum viscosity and the product consistency) takes place, the peach juice consistency is increased, suggesting that the changes in the serum composition and particle disruption are the most important mechanisms in this process condition.

Changes between 10 and 15 min of ultrasonic processing:

After 15 min of processing, the microscopy shows higher cell disruption, with an almost absence of whole cells. As a consequence, a decrease in D[4,3], and D[3,2] (Figure 2.3), a reduction of the overall particle size and an increase in the particle dispersion (PSD) are observed (Figure 2.2). The increase in the particle size dispersion and the decrease in the polysaccharide molecular size in the serum could result in the reduction of the serum viscosity (Figure 2.7(B)) and juice consistency. On the other hand, a reduction on the particle size could result in the increase of the juice consistency, as well as the increase in the serum concentration could increase its viscosity. However, no changes were observed in the juice consistency (Figure 2.7(A)). Once again, a complex relationship among these mechanisms dictates the juice observed behaviour. The high structural modification on both serum and particles, and the consequent new particle-particle and particle-serum interactions, forms aggregates, becoming the peach juice more thixotropic (Figure 2.8). Under these conditions it is suggested that the Brownian motion dominates. Where, the colloidal systems are characterized by the presence of particles much larger than the size of molecules of the dispersing medium (serum), but it is small enough so that the Brownian movement is not interfered. Therefore, although the juice consistency is not changed, its behaviour at low shear changes is, increasing the thixotropy.

Summarizing, the present description shows an important academic contribution, as this study described and demonstrated that the juice properties changes are not simply proportional to the processing time, but show a complex

behaviour due to the balance among several mechanisms. Further, as the juice shows an increasing in the pulp sedimentation stability, an improving in its consistency and a minimal change in its appearance – all of them high desired results from a commercial/industrial point of view (it is also important to highlight that any industrial application would needs a scale up study). For example, according to the purpose, the juice can be processed for 6 min (considering the evaluated conditions), obtaining any sedimentation with similar consistency to the unprocessed juice (if the present consistency is desirable). However, the juice can be processed for 10 min, where the pulp sedimentation is also avoided, but ensuring higher consistency (which is, in general, desirable). In conclusion, the ultrasound technology has proven to be a powerful tool to enhance the fruit juice properties and stability.

2.4   Conclusions

The present work evaluated the effect of the ultrasound technology on the physical properties and stability of peach juice. The ultrasonic processing causes the disruption of the remained suspended particles, but in a pattern involving the breakage of the pre-existing disrupted cells, a partial rupture of the cell wall with the releasing of intracellular constituents and finally the disruption of whole cells, resulting in different changes of suspended particle composition, size and distribution. The serum phase composition and properties are also changed during processing, due to both the release of intracellular constituents and polysaccharide degradation. As a consequence, the observed juice properties show a complex behaviour in relation to the ultrasonic processing time. While the pulp sedimentation is highly reduced by the process, both juice consistency and serum cloudiness (turbidity) showed an increase, followed by a decrease and then a new increase in relation to the processing time (being this firstly described). The colour of the processed samples showed a slight increase in lightness (L*) parameter, with small changes during storage. Therefore, the obtained results indicated that the ultrasound technology can be used to improve the physical properties of peach juice, increasing the stability to sedimentation and sensory acceptance, reducing the addition of additives such as hydrocolloid, with negligible changes in its colour. In fact, the obtained results show both academic and industrial/commercial relevance.

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Acknowledgments

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3   THE ULTRASOUND TECHNOLOGY FOR MODIFYING ENZYME ACTIVITY*

Abstract

Enzymes are protein complexes compounds widely studied and used due to their ability to catalyze reactions. The food processing mainly aims the inactivation of enzymes due to various undesirable effects. However, there are many processes that can be optimized by its catalytic activity. In this context, different technologies have been applied both to inactivate or to improve the enzymes efficiency. The Ultrasound technology emerges as an alternative mainly applied to achieve the enzyme inactivation. On the contrary, very few investigations show the ability of this technology under certain conditions to achieve the opposite effect (i.e. increase the catalytic activity of enzymes). The objective of this study was to correlate the ultrasonic energy delivered to the sample (J/mL) with the residual enzymatic activity and explain the possible mechanisms which results in the enzymatic activation/inactivation complex behavior. The activity of POD in coconut water was evaluated as a model. The enzymatic activity initially increased, followed by reduction with a trend to enzyme inactivation. This complex behavior is directly related to the applied ultrasonic energy and their direct mechanical effects on the product, as well as the effect in the enzymatic infinite intermediate states and its structural conformation changes. The obtained results are useful for both academic and industrial perspectives.

Keywords: Peroxidase (POD); Enzymatic activity; Enzymatic structural conformation; Ultrasound technology; Coconut water

3.1   Introduction

The enzyme-catalysed reactions are important in pharmaceutical, chemical, non-alimentary and alimentary industry. It is important during the processing and preservation of food, since the activity of enzymes includes undesirable reactions such as browning, rancidity, discoloration, loss of texture, among others. In this case, it is necessary to inactivate the enzymes. However, there are enzymes that catalyse desirable reactions, such as that used for hydrolysis, clarifying or to soften meat. Traditional thermal methods such as sterilization, pasteurization, precooking or blanching are the methods most known and used by the food industry for the inactivation of enzymes, although many new technologies are also studied and applied. Biotechnological techniques and also some innovative technologies are also used to improve and increase the enzymatic efficiency.

In fact, the ultrasound technology is gaining importance. There are many studies of ultrasound application to achieve enzyme inactivation, such as for lipases, proteases, peroxidase (POD), polyphenoloxidase (PPO), polygalacturonase (PG), pectinesterase, pectinmethylesterase (PME), ascorbate peroxidase (APx) (VERCET et al., 2001; TIWARI et al., 2009; COSTA et al., 2013; HUANG et al., 2015). On the other hand, few works demonstrate the capacity of these technology to enhance the enzyme activities.

It is difficult to identify the specific enzyme mechanism during sonication, which could be due to a singular or combination of several chemical and physical effects occurring simultaneously (RAWSON et al., 2011). In this work, the coconut water is considered as a model product to be investigated because despite different studies that have been performed using different technologies (such as the conventional thermal process (MURASAKI-ALIBERTI et al., 2009; FONTAN et al., 2012; TAN et al., 2014b), membranes (NAKANO et al., 2011; DAS PURKAYASTHA et al., 2012) , use of additives (ABREU; FARIA, 2007; PEREIRA; FARIA; PINTO, 2013), microwave (MATSUI et al., 2007, 2008) and ultraviolet radiation (AUGUSTO et al., 2015), problems related with its enzyme stability (POD and PPO) are still observed.

Since the ultrasound technology application in coconut has not been studied yet, this work evaluated the peroxidase (POD) enzymatic behaviour during the ultrasonic processing, considering two equipment with different frequency and acoustic intensity.

3.2   Material and method

3.2.1   Raw material preparation

Coconut water (pH: 5.0 ± 0.4; °Brix: 5.0 ± 0.8) was obtained of green coconuts from the local market (CEASA/Ceagesp, SP, Brazil). After cleaning and sanitizing, the mesocarp of coconuts was drilled with a special knife to extract their water. The water was filtered to remove particulate matter. The product obtained of different fruits was mixed, portioned and rapidly frozen (~-20 °C) for all future processes and analyses.

3.2.2 Ultrasound processing

The coconut water was processed using two ultrasonic equipment (probe and bath). The sample (105 mL) was processed for 20 min using an ultrasonic probe (ECO-SONIC, QR1000 Model, Brazil) with a frequency of 20 kHz, acoustic density 286 W/L, 1.26 cm^2 titanium probe (keeping it at 3 mm depth in the samples). Also, the coconut water (1700 mL) was processed for 3 hours using an ultrasonic bath ((UNIQUE, USC-1400 Model, Brazil) with a frequency of 40 kHz and 28 W/L of acoustic density. These conditions were selected after pre-evaluations. In order to control the process temperature (23.7 ± 2 °C), heat exchangers with cold water circulation were used. Along the processing period, samples of 3.5 mL were taken out in order to obtain the enzyme activity. All processes were carried out in three replicates.

The absolute ultrasonic power P (W) and acoustic energy density (W/L) was determined calorimetrically (O'DONNELL et al., 2010; FONTELES et al., 2012). The ultrasonic energy consumption (U_{ec}) was calculated according $U_{ec} = P.t_{US}$ (J/mL), where t_{US} is the ultrasound processing time.

3.2.3 Enzyme activity evaluation

The enzyme activity assays were determined in duplicate for each sample at 24 ± 1 °C and pH 6.0. This condition was selected to be the optimum pH and temperature of the coconut water enzymes (MATSUI et al., 2007, 2008; MURASAKI-ALIBERTI et al., 2009; DAS PURKAYASTHA et al., 2012; TAN et al., 2014a).

The pH 6.0 was ensured using a buffer solution (McIlvaine's buffer), which was prepared using citric acid ($C_6H_8O_7$) (Synth, São Paulo) and sodium phosphate dibasic (Na_2HPO_4) (Synth, São Paulo). For this purpose, a proportion of 1.00 mL of $C_6H_8O_7$ (0.2M): 2.45 mL of Na_2HPO_4 (0.4 M) was mixed.

The POD activity was evaluated using pyrogallol ($C_6H_6O_3$) (Sigma-Aldrich, India) as the substrate as described by Falguera et al. (2013) and Augusto et al. (2015), with a few modifications. In each assay, 1.5 mL of coconut water, 1 mL of buffer solution at pH 6.0 and 320 μL of 5% (m/v) pyrogallol solution was mixed in a quartz cuvette with a 1 cm light path. The mixture of all reagents was used as reference solution (0,000 absorbance). Then, 160 μL of hydrogen peroxide (H_2O_2) (Synth, São

Paulo) 0.147 M solution was added and mixed, which starts the reaction. The increase in the solution absorbance (Abs) at 420 nm was measured every 20 s for 10 min using a UV–Vis spectrophotometer (Uvmini-1240, SHIMADZU, Japan).

As described by Augusto et al. (2015), the increase of absorbance at 420 nm in relation to the reaction time shows a downward concave shape curve, which could be described by a composite exponential function (eq. 3.1).

$$Abs_{(t_{Abs})} = Abs_\infty - (Abs_\infty - Abs_0).e^{(-k_{Abs}.t_{Abs})} \tag{3.1}$$

Where $Abs_{(t_{Abs})}$ is the sample absorbance at 420 nm at any time t_{Abs}, Abs_0 is its initial absorbance, Abs_∞ is the maximum absorbance at the equilibrium and k_{Abs} is the kinetic parameter.

The enzyme activity (A) was then defined as the maximum reaction rate, which is observed when $t_{Abs} = 0$, thus being defined by:

$$A = \left(\frac{dAbs(t_{Abs})}{dt_{Abs}}\right)_{t_{Abs}=0} = (Abs_\infty - Abs_0).k_{Abs} \tag{3.2}$$

The parameters for each model with a confidence level of 95% were obtained by regression using the Levenberg-Marquardt algorithm in Statistica 13 (StatSoft, USA) software. In order to obtain the kinetics of POD, the relative activity $A_{(tUS)}/A_0$ was evaluated during the ultrasound processing time.

3.3 Results and discussion

All the previously studied methods have demonstrated an effective reduction of the enzyme activities in coconut water (conventional thermal process, ultrafiltration, additives addition, processing with microwave and ultraviolet radiation). However, the enzymes naturally present in coconut water showed a higher resistance when compared to those added to the sterilized medium or those added to model solutions (MATSUI et al., 2007; AUGUSTO et al., 2015).

Figure 3.1 shows the peroxidase (POD) residual activity of coconut water processed using the ultrasound bath and the ultrasound probe. It is observed that, at the same level of energy added to the system, both activation and inactivation were achieved, each one in one system.
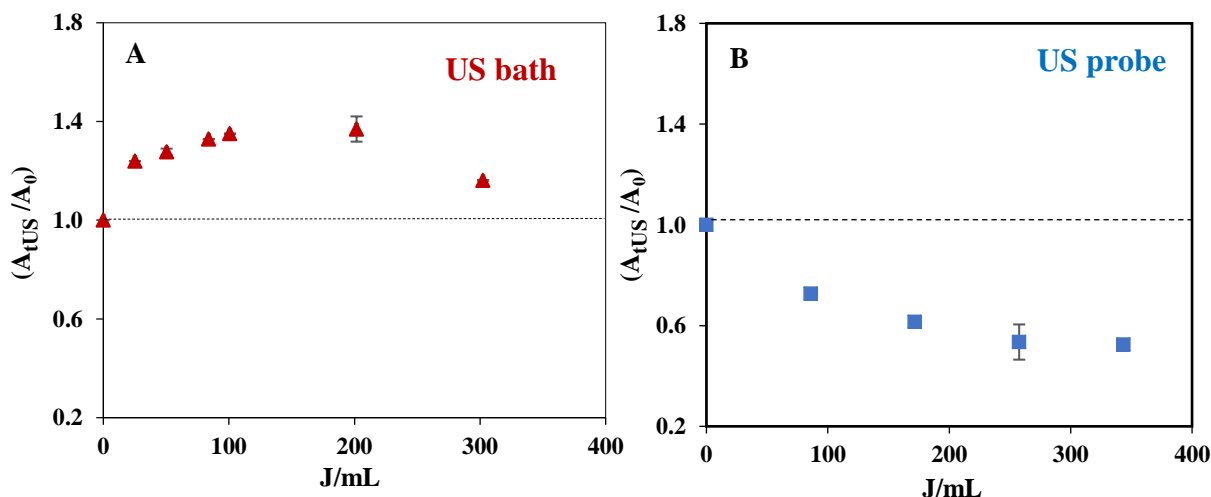

Figure 3.1 – POD residual activity after ultrasound processing: process with ultrasound bath for 3 h (A) and process with ultrasound probe for 20 min (B). Horizontal discontinuous line is the limit for being enzyme activation/inactivation. Vertical bars are the standard deviation (α=0,05)

The activation behaviour can be observed when high frequencies (40 kHz in the case of US bath) and low power (WU; LIN, 2002) are used; therefore, long processing times (> 3 h) are required to start inactivation. On the other hand, when low frequencies (20 kHz in the case of US probe) and high acoustic intensities were used, short times are required to achieve inactivation (and the activation period can be too short, that it is difficult to be seen). In fact, partial inactivation effects (i.e. the enzyme inactivation is not achieved completely) were reported by Silva et al. (2015) in apple. Further, variations of increase or decrease in enzyme activity was observed under specific operating conditions (time, ultrasound intensity, temperature) (FONTELES et al., 2012; ENGMANN et al., 2014). For example, an increase in the enzyme activity was observed for POD with increasing processing time, and for PPO at higher temperatures (60 °C) in apple (SILVA et al., 2015). On the other hand, PPO inactivation was reduced as ultrasonic frequency and treatment time were increased in mulberry (ENGMANN et al., 2014), indicating an inverse relationship. Therefore, a general conclusion cannot be specified, as the properties of both product (pH, activity of water/vapour pressure,

ionic strength, composition) and process (kind of equipment, volumetric power, frequency, intensity, amplitude, reactor geometry and waves distribution) influence the enzyme activity.

The ultrasonic mechanisms that change the enzyme activity are specific to the enzyme under investigation and depends on its amino acid composition and the conformational structure (ÖZBEK; ÜLGEN, 2000). It is difficult to identify the specific enzyme inactivation mechanism during sonication, which could be due to a singular or combination of several chemical and physical effects occurring simultaneously (RAWSON et al., 2011), resulting in multiple responses and possibilities. Table 3.1 reports the major factors that can affect the enzyme activity during ultrasonic processing.

Since the enzyme have multiple responses, it is suggested that it can have infinite intermediate states (related with each spatial configuration associated with each value of internal energy of the system) during processing and that each intermediate state can result in an increase ($A_{(tUS)}/A_0$ >1), decrease ($A_{(tUS)}/A_0$ <1) or even the same ($A_{(tUS)}/A_0$ = 1) activity. Then, the enzymes need energy (ε) to pass from one to another state (eq. 3.3) and each energy quantum added to the system, results in a conformational change, which can change the enzyme activity. However, most of the works consider only two to four possibilities, which can be seen as a simplification (with good or bad description of the experimental data). Even so, it is important to expand the possibilities in order to better interpret the enzyme activity data.

$$\mathbf{E_0} \underset{\longleftarrow}{\overset{\varepsilon_{(0)\text{-}(1)}}{\longrightarrow}} \mathbf{E_1} \underset{\longleftarrow}{\overset{\varepsilon_{(1)\text{-}(2)}}{\longrightarrow}} \cdots \underset{\longleftarrow}{\overset{\varepsilon_{(n\text{-}1)\text{-}(n)}}{\longrightarrow}} \mathbf{E_n}$$
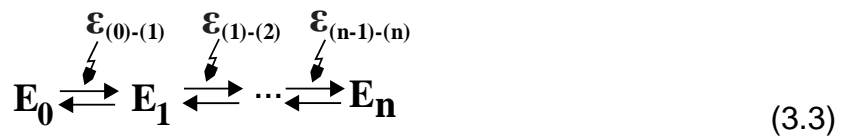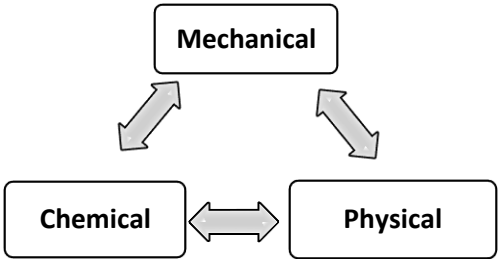
(3.3)

Table 3.1 – Major factors that can affect the enzyme activity during ultrasonic processing

Ultrasound factor

Mixing, particle collision, high shear rates and strong micro-streaming, ultrasonic waves propagation, sample cavitation, severe rise in temperature and pressure, sonolysis of water molecules (ERCAN; SOYSAL, 2011; TEREFE et al., 2009; VERCET et al., 2001).

Possible effect	Activity response
Phenolic production as part of stress responses to a mechanical stimulus (WU; LIN, 2002).	Increase
Collisions promotes enzyme – substrate contact.	Increase
Activation of latent isoenzymes (ENGMANN et al., 2014).	Increase
Dissociation of enzyme aggregates (LÓPEZ et al., 1994).	Increase
Changes in the three-dimensional structure (CRUZ; VIEIRA; SILVA, 2006).	Increase/decrease
Conformation changes in the active site three-dimensional structure, enzyme–substrate interaction ((CRUZ; VIEIRA; SILVA, 2006).	Increase/decrease
Molecular unfolding, causing the exposure of more hydrophobic groups and regions from inside to the outside (FENG et al., 2016).	Increase/decrease
Disruption of intra- and intermolecular substrate molecule interactions (BARTON; BULLOCK; WEIR, 1996).	Increase/decrease
Inactivation of sensible isoenzymes fraction.	Decrease
Splitting of prosthetic group of hemoproteins (WEISSLER, 1960); the same could occur in holoenzymes (LÓPEZ et al., 1994), such as peroxidase.	Decrease
Protein denaturation (TEREFE et al., 2009).	Decrease

The results obtained in the present work suggest the applicability of the ultrasound technology to increase or decrease the enzyme activity. The increase in enzyme activity is desired in many industrial process which use enzymes for catalyse reactions such as to enhance the enzyme activities using supersaturated solutions (LEE et al., 2008), improve the enzymatic reaction rate (BARTON; BULLOCK; WEIR, 1996; SAKAKIBARA et al., 1996) and accelerate enzymatic synthesis (XIAO et al., 2005). In fact, some recent studies used the ultrasound to improve the enzymatic efficiency such as in hydrolysis reactions by lipase (WAGHMARE; RATHOD, 2016) where the ultrasound considerably reduced the reaction time as compared to conventional reaction, or to improve the enzymatic activity of immobilized papain (FENG et al., 2016). Consequently, the present work highlights the broad use of ultrasound technology for food processing.

3.4 Conclusions

Enzymes can present multiple states as response of processing. This complex behaviour depends on the system composition, enzyme conformation and processing properties. During ultrasonic processing, physical, mechanical or chemical factors directly affect the product and the three-dimensional structure of the enzyme. In this study, it was shown that the ultrasound technology has the ability to increase or decrease the enzyme activity, depending on the energy applied to the product, the frequency and other properties related with the ultrasound equipment design. This information can thus be exploited to both objectives (i.e. improving catalytic activity of enzymes or promote the inactivation and/or enzymatic sensitization), expanding the possible uses of ultrasound industrially.

Acknowledgments

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4 USING ULTRASOUND TECHNOLOGY FOR THE INACTIVATION AND THERMAL SENSITIZATION OF PEROXIDASE (POD) IN COCONUT WATER*

Abstract

Green coconut water is a unique beverage due to its nutritional properties and sensorial qualities; however, despite the different technologies already studied, it is still challenging to improve its enzymatic stability. This study evaluated the use of ultrasound technology (US) for inactivating/sensitizing coconut water peroxidase (POD). The effect of both US application alone and as a pre-treatment to subsequent thermal process (US-TT) was evaluated. The enzyme activity during US processing was reduced by approximately 27% after 30 min at 286 W/L (20 kHz), demonstrating the high resistance of POD. The thermal inactivation kinetics did not show first order behaviour, and the Weibull distribution function was used to describe it under non-isothermal conditions, taking into account the whole temperature history of the sample. The differences obtained in the Weibull parameters indicate that the enzyme with US pre-treatment became sensitized to heat. Further, the use of US resulted in more uniform heat resistance (the US-TT samples require approximately 46% less exposure time at 85 °C to achieve 99% of POD inactivation). The results suggest that US is a good technology for sensitizing enzymes before a subsequent thermal process (even for an enzyme with high thermal resistance such as the coconut water POD). Therefore, the use of this technology could decrease the undesirable effects of long times and/or the high temperatures of the conventional thermal processing.

Keywords: Ultrasound; Thermal processing; Coconut water; Peroxidase; Inactivation kinetics

4.1   Introduction

Green coconut water is a tropical beverage obtained from the immature coconut fruit (*Cocos nucifera L.*) (DEBMANDAL; MANDAL, 2011; PRADES et al., 2012), with an increasing demand not only due to its sensory properties, but also due to its nutritional characteristics. It contains a remarkable content of salts and minerals, such as potassium, sodium, chloride, magnesium and also of sugars (NEPA-UNICAMP, 2011), being considered a drink with great potential for rehydration (PRADES et al., 2012) and health (YONG et al., 2009). In addition, coconut water contains other important compounds such as free amino acids (serine, glycine, histidine, tyrosine, phenylalanine, isoleucine and leucine (OVALLES et al., 2002), vitamins of the B-complex and enzymes (phosphatase, catalase, dehydrogenase, diastase, peroxidase, polyphenoloxidase, RNA polymerases) (YONG et al., 2009; DEBMANDAL; MANDAL,

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2011; PRADES et al., 2012). In fact, the presence of enzymes is an important factor for industrialization since their activity results in undesirable changes, such as the development of pinkish and dark brown colours (PENHA; CABRAL; MATTA, 2005).

The shelf life of green coconut water after extraction depends on the preservation methods used. These methods should aim to inhibit the enzyme activity while ensuring the microbiological quality and maintaining the original sensory characteristics (PENHA; CABRAL; MATTA, 2005). Although the thermal process is efficient from a microbial point of view, the relative high resistance of the native enzymes demands a process severity that results in sensorial and nutritional drawbacks (CAMPOS et al., 1996; PENHA; CABRAL; MATTA, 2005). Consequently, although the thermal processing of coconut water combined with the use of preservatives is commercially used, its negative impact on sensorial and nutritional properties motivates the study of alternatives.

Conventional and non-conventional alternatives have already been studied and even applied, but they have their own drawbacks. Coconut water freezing is effective, but it requires a cold chain until the moment of consumption. Microfiltration (MAGALHÃES et al., 2005) removes the enzymes, but it also removes some nutrients (REDDY; DAS; DAS, 2007; NAKANO et al., 2011). The use of preservatives and carbon dioxide addition were also studied (PEREIRA; FARIA; PINTO, 2013), but they cause a change of flavour, decreasing product acceptability. High hydrostatic pressure technology has already been commercially adopted for this purpose, but it is not effective for enzyme inactivation (for instance, there is a commercial product whose characteristic pink colour is due to the enzymatic action (UNOCO, 2016)). Ultraviolet radiation was proposed to be effective in a model solution (AUGUSTO et al., 2015), but there is no information regarding the real product sensorial properties.

Ultrasound (US) technology, albeit promising, has not been used for coconut water enzyme inactivation yet, even though this technology has already been applied to coconut water combined with supercritical carbon dioxide for microbial inactivation (CAPPELLETTI; FERRENTINO; SPILIMBERGO, 2014). Furthermore, the sonication alone is not very effective in inactivating microorganisms in food, but it can be coupled with high pressure (manosonication) or mild heat (thermosonication) to be effective (ZINOVIADOU et al., 2015).

US technology can affect the enzyme structure due to physical and/or chemical mechanisms (ROJAS; TREVILIN; AUGUSTO, 2016). Such mechanisms can affect the

native conformation of the enzyme that depends on hydrophobic interactions, hydrogen bonding, van der Waals interactions, ion paring, electrostatic forces and steric constraints to stabilize the three-dimensional molecular structure of globular proteins (CHEMAT; ZILL E; KHAN, 2011). Each energy quantum added to the system results in a conformational change, which can increase or decrease enzyme activity (ROJAS; TREVILIN; AUGUSTO, 2016), requiring a case-by-case study in order to understand it. Moreover, US technology alone is generally less efficient for enzyme inactivation than conventional thermal treatment. However, thermosonication is quite effective, for example, in inactivating peroxidase from horseradish (DE GENNARO et al., 1999), which suggests that this technique has interesting possibilities in food processing technology. Another less studied possibility, which requires a simpler industrial plant, is using US technology as a pre-treatment to thermal processing to thermally sensitize the enzymes, allowing a less severe thermal processing, instead of conducting both processes at the same time (thermosonication). This possibility is particularly interesting since the ultrasonic efficiency decreases with increasing temperature (SAEEDUDDIN et al., 2015).

The present work evaluated the use of US technology for inactivating peroxidase (POD) in green coconut water, as well as a pre-treatment to reduce the thermal resistance of this enzyme. For this, considering the complexity of enzyme inactivation, kinetics other than the first-order model was considered. Furthermore, a non-isothermal evaluation was carried out to take into account the whole temperature history of the sample instead of assuming instantaneous temperature changes.

4.2   Material and methods

4.2.1   Raw material

One batch of green coconut was obtained from a local market (CEASA/Piracicaba, SP, Brazil). After cleaning and sanitizing, the mesocarp of the coconuts was drilled with a special knife to extract their water. The water was then filtered (standard paper filter) to remove particulate matter. The product obtained was mixed, portioned in volumes of 110 mL, placed in bottles and rapidly frozen (ca. -20 °C) in a freezing chamber. The mixture had the following characteristics: °Brix (4.20),

pH (4.68), total solids (4.83%), titratable acidity expressed as citric acid percent (1.35%), total reducing sugars (3.94%). The samples were thawed using a refrigerator (Consul 340, Brazil) at 4 °C before processing.

4.2.2 Ultrasound processing

Coconut water samples (100 mL) were processed using an ultrasonic tip (nominal power of 1000 W, 20 kHz - ECO-SONIC, QR1000 Model, Brazil) with a 1.26 cm2 titanium probe (keeping it at 3 mm depth in the samples) and applying an acoustic density of 286 W/L (by adjusting the equipment to 50% of its capacity). In order to control the process temperature (ca. 25 ºC), the samples were placed in a jacketed vessel with water circulation (12 ºC), using a heat exchanger connected to a thermostatic bath (Solaris 100, SP, Brazil). The samples were processed for 30 min (pausing for about 2 min every 5 min during processing to avoid overheating the ultrasound equipment), and at each time of 0, 5, 10, 15, 20 and 30 min, an aliquot of 3.5 mL was removed and immediately evaluated. This process was conducted in at least three replicates and the conditions were selected after preliminary tests, ensuring that the sampling effect was negligible for the acoustic power.

4.2.3 Thermal processing

Both samples, without pre-treatment (hereinafter named TT samples) and pre-treated with ultrasound for 30 min (condition selected based on the results, hereinafter named US-TT samples), were thermally processed for evaluation of the inactivation kinetics.

For each test, a sample of 5 mL was placed in glass tubes with small diameter and thin walls (100x10x0.6 mm – height x external diameter x wall thickness, respectively). The thermal process was carried out at three process temperatures (80, 85 and 90 °C) and six holding times for each temperature, using a thermostatic water bath with orbital shaking at 150 RPM (Marconi, DUBNOFF MA 095/CFRE, Brazil – it can maintain the process temperature (T_p) with a precision of ±1 ºC by PID control). Shaking assured temperature homogeneity in the samples. During the thermal process, the temperature history of each sample was monitored with thermocouples

(1.0 mm wire diameter; copper- constantan-type T thermocouple) connected to a data logger (ALMEMO 2890-9, AHLBORN, Germany), with a sampling rate of 1 s^{-1}. After the desirable holding time, the samples were quickly cooled in an ice-water bath until temperatures below 25 °C were obtained, thus avoiding further thermal inactivation. Then, the enzyme activity was determined. All the experiments were performed in triplicate.

4.2.4   Enzyme activity assay

As the peroxidase (POD) is the most resistant enzyme in green coconut water (MURASAKI-ALIBERTI et al., 2009), it is a good processing target, being the focus of the present work.

The enzyme activity, for each aliquot removed during the processing, was determined in duplicate at 24 ± 1 °C and pH 6.0. This condition was selected to be the optimum pH and temperature of the coconut water enzymes (MATSUI et al., 2007; MATSUI et al., 2008; MURASAKI-ALIBERTI et al., 2009; DAS PURKAYASTHA et al., 2012; TAN et al., 2014).

The desired pH was obtained using a buffer solution (McIlvaine's buffer) prepared with citric acid ($C_6H_8O_7$) and sodium phosphate dibasic (Na_2HPO_4) (Synth, São Paulo, Brazil). For this purpose, a proportion of 1.00 mL of $C_6H_8O_7$ (0.2 M) to 2.45 mL of Na_2HPO_4 (0.4 M) was mixed.

The POD activity was evaluated using pyrogallol ($C_6H_6O_3$) (Sigma-Aldrich, India) as the substrate as described by Augusto et al. (2015) and by Falguera et al. (2013) with a few modifications. In each assay, 1.5 mL of coconut water, 1.0 mL of buffer solution at pH 6.0 and 320 μL of 5% (m/v) pyrogallol solution were mixed in a quartz cuvette with 1.0 cm of light path. The mixture of all reactants was used as a reference solution (0.000 absorbance). Then, 160 μL of hydrogen peroxide (H_2O_2) (Synth, São Paulo, Brazil) 0.147 M solution was added and mixed to start the reaction. The increase in the solution absorbance (*Abs*) at 420 nm was measured every 20 s for 10 min using a UV–Vis spectrophotometer (Uvmini-1240, SHIMADZU, Japan).

As described by Augusto et al. (2015), the increase of absorbance at 420 nm in relation to the reaction time shows an increasing curve with concave shape, which could be described by a composite exponential function (eq. (1), where $Abs_{(t_{Abs})}$ is the

sample absorbance at 420 nm at any time t_{Abs}, Abs_0 is its initial absorbance, Abs_∞ is the maximum constant absorbance and k_{Abs} is the kinetic parameter).

$$Abs_{(t_{Abs})} = Abs_\infty - (Abs_\infty - Abs_0).e^{(-k_{Abs}.t_{Abs})} \tag{4.1}$$

The enzyme activity (A) was then defined as the maximum reaction rate, which is observed when $t_{Abs} = 0$ (eq. 4.2).:

$$A = \left(\frac{dAbs(t_{Abs})}{dt_{Abs}}\right)_{t_{Abs}=0} = (Abs_\infty - Abs_0).k_{Abs} \tag{4.2}$$

The parameters were obtained by regression using the Levenberg-Marquardt algorithm with a confidence level of 95% in Statistica 13 (StatSoft, USA) software.

4.2.5 Coconut water absorbance spectrum

The absorbance spectra of the coconut water were evaluated in the UV–Vis range as a function of the processing time (ultrasonic and thermal). For this, a 3 mL sample was added to a quartz cuvette with 1.0 cm of light path for evaluation in the same spectrophotometer used in the enzymatic assay. The solution absorbance (Abs) was then measured for wavelengths between 235 and 295 nm.

4.2.6 Enzymatic inactivation kinetics and statistical analysis

The inactivation kinetics was evaluated based on the Weibull Model, considering the actual non-isothermal conditions (Figure 4.3).

Equation 3 shows the Weibull function (WEIBULL, 1951; SMITH, 1991) applied for enzymatic inactivation. In this equation, A_t/A_0 is the relative enzymatic activity at process time t, β is a parameter that indicates the shape of the inactivation curve (on a semi-logarithmic plot, $\beta > 1$ indicates a convex shape and $\beta < 1$, a concave shape; a log linear shape is a special case when $\beta = 1$) and δ_T represents the time for the first decimal reduction at a given temperature T. This parameter is distinguished from the

conventional D_T value, which is derived from the first-order kinetic model and represents the time to achieve a tenfold decrease in the enzymatic activity (i.e. $\delta_T = D_T$ only in the special case when β =1) (MAFART et al., 2002). More details are provided in the "results and discussion" section.

$$\log\frac{A_t}{A_0} = -\left(\frac{t}{\delta_T}\right)^\beta \tag{4.3}$$

The classical relation used to describe the dependence of δ_T on temperature T (MAFART et al., 2002), based on the Bigelow model (BIGELOW et al., 1920), was applied as shown in eq. (4.4). Parameter z is the temperature change required to cause a tenfold change in δ_T and δ_{Tr} is the time for the first decimal reduction at a reference temperature T_r, which must be within the experimental temperature range. The reference temperature T_r was set at 85 °C in this work.

$$\log\frac{\delta_T}{\delta_{Tr}} = \frac{T_r-T}{z} \tag{4.4}$$

Since the heating and cooling of the sample are not instantaneous, as can be seen in the example in Figure 4.1, the lethality function $L_t(t)$ defined in eq. (4.5) was used to take into account the whole temperature history $T(t)$, assuming homogeneous temperature due to the stirring. By integrating this function with respect to time, the equivalent time or integrated lethality (F_{T_r}) can be obtained (eq. (4.6)). Variable F_{T_r} is the equivalent processing time at T_r if instantaneous heating and cooling were possible, yielding the same inactivation as the actual process with temperature history $T(t)$ (TAJCHAKAVIT; RAMASWAMY, 1997; MURASAKI-ALIBERTI et al., 2009).

$$L_t(t) = 10^{\frac{T(t)-T_r}{z}} \tag{4.5}$$

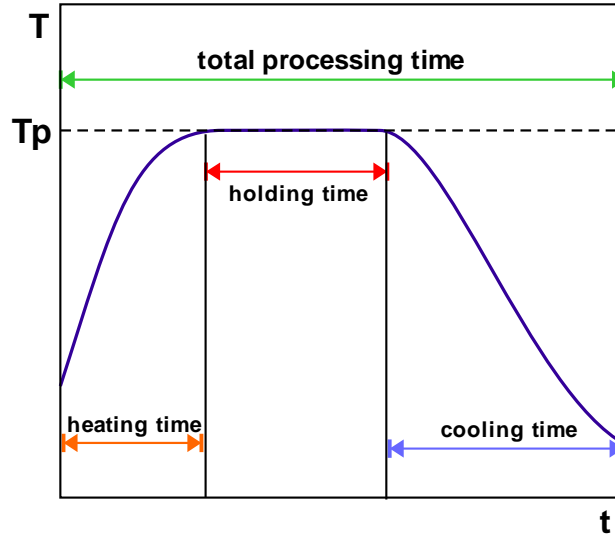$$F_{T_r} = \int_0^t L_t(t)dt = \int_0^t 10^{\frac{T(t)-T_r}{z}}dt \tag{4.6}$$

Figure 4.1 – Representation of the sample temperature history during non-isothermal processing, where Tp is the desired process temperature

The Weibull kinetic model in eq. (4.3), can thus be rewritten using the concept of F_{T_r}:

$$\log \frac{A_t}{A_0} = -\left(\frac{F_{T_r}}{\delta_{Tr}}\right)^{\beta} \tag{4.7}$$

Later, the effect of the sonication prior to the thermal processing was modelled assuming that this pre-treatment reduces the initial enzymatic activity A_0 by a factor $\lambda \in [0,1]$, as shown in eq. (4.8), where A_0' is the enzymatic activity after sonication:

$$A_0' = \lambda A_0 \tag{4.8}$$

The Weibull kinetic model can thus be rewritten as eq. (4.9) to take into account the sonication effect in the residual enzymatic activity of the ultrasound pre-treated sample subject to thermal processing.

$$\frac{A_t}{A_0} = \lambda 10^{-\left(\frac{F_{T_r}}{\delta_{Tr}}\right)^{\beta}} \tag{4.9}$$

The parameters of the kinetic model in eq. (4.9) (β, δ_{Tr}, z and λ) were iteratively adjusted by minimizing the sum of squared errors (SSE in eq. (10)) between the experimental and the predicted residual activities for the n experiments and taking into account all process temperatures and replicates. It was assumed that the shape factor β was independent of the processing temperature and that λ was constant, since the sonication conditions were the same for all pre-treated samples (286 W/L, 20 kHz, 30 min, 25 °C). A generalized reduced gradient algorithm was used, implemented in the 'Solver' tool of software Excel 2010 (Microsoft, USA). The different initial guesses of the four parameters were tested to detect possible convergence to local optima. To report the fit criteria of the model, we considered the minimized SSE, the coefficient of determination R^2 and the distribution of the residuals.

$$SSE = \sum_{i=1}^{n} \left(\left(\frac{A_t}{A_0} \right)_{pred} - \left(\frac{A_t}{A_0} \right)_{exp} \right)_i^2 \qquad (4.10)$$

4.3   Results and Discussion

4.3.1   Ultrasound processing and POD inactivation

Figure 4.2 shows the residual POD activity and the UV–Vis absorbance spectrum of the coconut water during ultrasonic processing. After 30 min of ultrasonic processing under the applied conditions (286 W/L, 20 kHz, 25 °C), the residual activity was 0.73 ± 0.07, i.e. approximately 27% of inactivation was reached. This demonstrates that long processing times and/or high intensities are required to achieve an important enzyme inactivation level, as also observed by Tiwari et al. (2009); Costa et al. (2013); Huang et al. (2015) and O'Donnell et al. (2010). Although the result apparently indicates that US technology is not efficient for the POD inactivation in coconut water, it is highlighted that this technology can indeed be effective when using high power equipment.

Multiple factors influence enzyme inactivation during US processing and they can be summarized as external factors, which include ambient conditions and equipment characteristics (temperature, time of process, power density or intensity and

frequency) and intrinsic factors, which depend on the food matrix characteristics, composition besides target enzyme and its characteristics.

Previous studies demonstrated that ultrasonic processing can cause protein structural changes that modify the enzyme activity. In fact, for native horseradish POD (which show high α-helix content - each peroxidase consists of ten α-helices surrounding the catalytic centre (WELINDER, 1992; DUNFORD, 2010)) the α-helices content decreased after sonication and the β-structure content increased in the induced aggregates (STATHOPULOS et al., 2004). Additionally, after sonication, the polyphenol oxidase structure became less rigid, due to the α-helices content decrease, also decreasing the enzyme activity (HUANG et al., 2016)

Nevertheless, the literature reports conflicting results regarding the POD inactivation by ultrasound, probably due to the variation of intrinsic and external factors. For example, at lower ultrasound frequencies, POD inactivation above 90% was reported in crude extract of tomato (ERCAN; SOYSAL, 2011) while an increase of intracellular POD activity was reported by Wu and Lin (2002) in a culture of root cells of *Panax ginseng* under ultrasound treatment at higher frequencies. Moreover, both increasing and decreasing POD activity were reported for the coconut water POD (ROJAS; TREVILIN; AUGUSTO, 2016), depending on the process conditions.

Different studies reported enzyme inactivation by ultrasound in vegetable products, such as in fresh-cut apples (JANG; MOON, 2011), sweet melon (LIU et al., 2016), pineapple juice (COSTA et al., 2013), mulberry juice (ENGMANN et al., 2014), cantaloupe melon juice (FONTELES et al., 2012) or orange juice (TIWARI et al., 2009). However, coconut water has a different structure: it is a liquid albumen, a solution of salts and sugar containing free nuclei with some dispersed proteins (enzymes) (YONG et al., 2009; DEBMANDAL; MANDAL, 2011; PRADES et al., 2012). Differently, fruit juices are composed of solid particles (whole cells, fragments of tissues and cells, cell walls) dispersed in a viscous serum (water solution of intracellular components such as sugar and salts). In these dispersions, the ultrasound causes an additional effect of cell disruption (ROJAS et al., 2016), thus changing the dispersed medium composition and properties, which can affect the enzyme activity.

Figure 4.2B shows the coconut water absorbance spectra affected by sonication time. It presents a minimum local peak near 250 nm and a maximum local peak close to 270 nm. The absorbance spectra observed is similar to those of aromatic amino

acids such as phenylalanine, tryptophan and tyrosine (LEHNINGER; NELSON; COX, 2005), as well as solutions of peroxidase (POD) and polyphenol oxidase (PPO) (AUGUSTO et al., 2015). Rosenheck and Doty (1961) evaluated the changes in the absorbance spectra of polypeptides and proteins under different conditions. For instance, the poly-L-lysine absorbance decreased when the molecule was denatured. A similar result was obtained for the POD evaluated in this work as can be seen in Figure 4.2B.
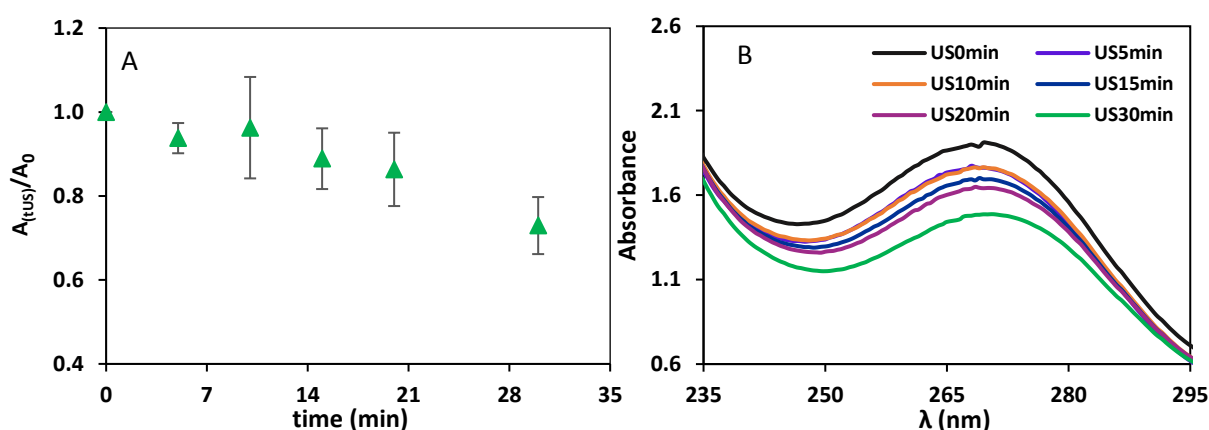

Figure 4.2 – (A) POD inactivation with ultrasonic processing at 25 ºC (residual activity versus US processing time), the vertical bars are the standard deviation. (B) UV–V is the absorbance spectrum of the coconut water for different ultrasonic processing times

Therefore, the importance of the ultrasound application in this study lies in the suggested changes in the POD structure, which influence their sensitivity to posterior processes, such as thermal processing, as will be discussed in the next section.

4.3.2 Analysis of the time-temperature history

Figure 4.3 shows the temperature history of the samples during the thermal processing at 80, 85 and 90 °C. It can be seen that even when using tubes with small diameter and wall thickness and holding a small volume under stirring, the process was still not ideally isothermal, especially for the case of short holding times (Figure 4.3 C1). On the other hand, it is clear that at longer processing times, the heating and cooling times were less important (Figure 4.3 A1 and B1). Note that if the process were

isothermal, then the points in Figures A2, B2, C2 would be located in the 45° dotted lines. However, this does not occur because of the contribution of heating and cooling times. Therefore, strict non-isothermal conditions were considered in the following evaluation.
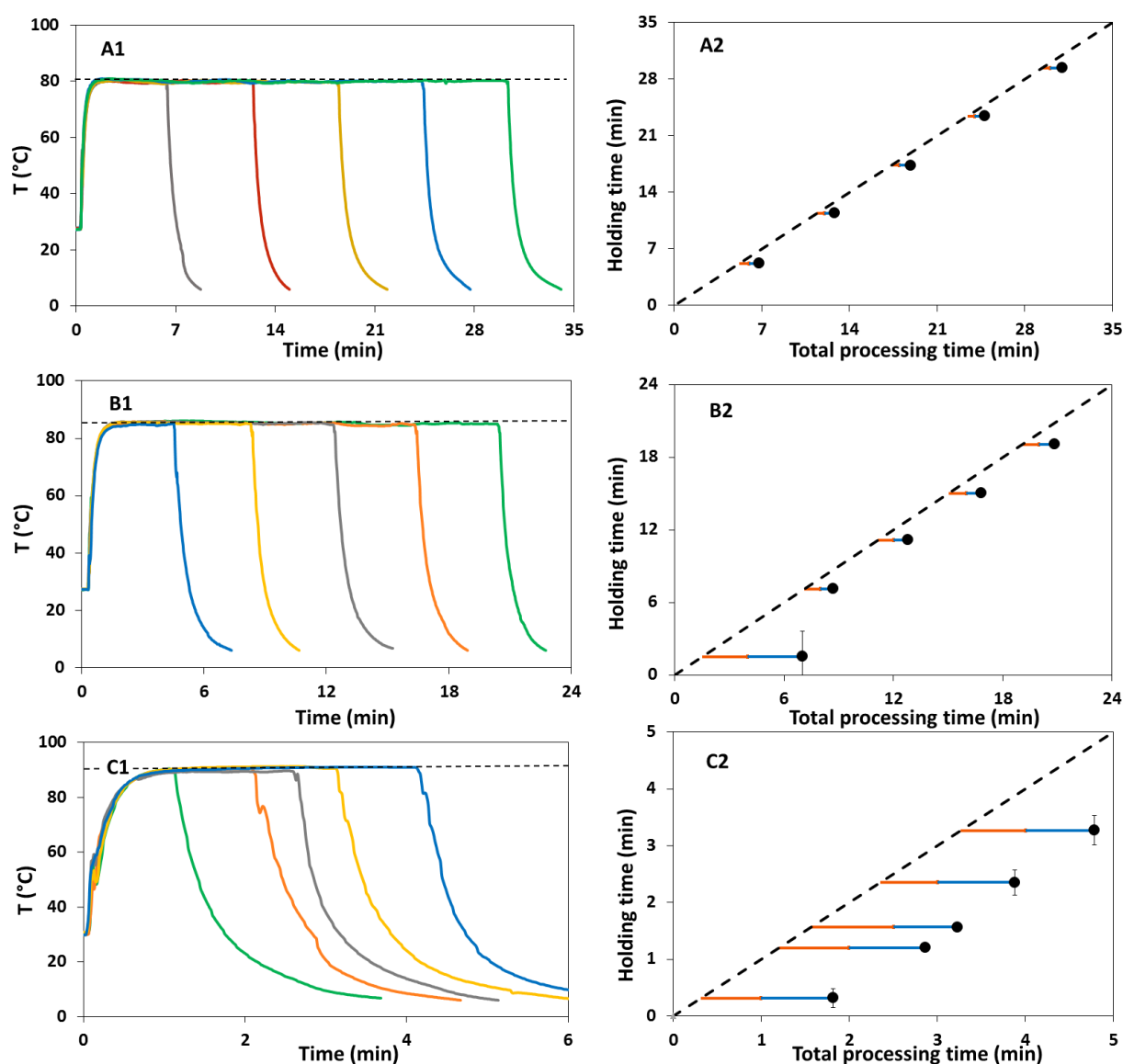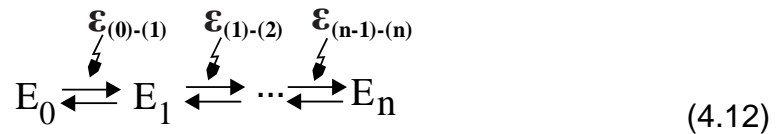

Figure 4.3 – Left: Sample temperature history during the thermal processes at 80, 85 and 90 °C (A1, B1, C1 respectively). Dashed lines represent the process temperature. Right: respective holding time versus total processing time (dots in graphs A2, B2 and C2). The total processing time was defined as $T > 27$ °C; the holding time was defined as T within 1 °C of the required temperature. The 45° dashed lines indicate isothermal process, while the orange and blue horizontal lines represent heating and cooling times, respectively

4.3.3 Effect of ultrasound pre-treatment on POD inactivation

Enzyme inactivation kinetics in food processing can be described by various models. The most used is the classical first order kinetics model, which describes the simplest behaviour of the enzyme inactivation mechanism and considers only a single fraction. Derivations of the first order model are also used, such as the biphasic inactivation model, which assumes two enzymatic fractions (labile and resistant) that contribute to the total activity (MURASAKI-ALIBERTI et al., 2009; AGUIAR; YAMASHITA; GUT, 2012; PRADES et al., 2012; AUGUSTO et al., 2015). However, plants naturally present multiple forms of the same type of enzyme (called isozymes) that catalyze the same reaction, but the isozymes may differ markedly in physicochemical and thermal resistance properties (SHANNON; KAY; LEW, 1966). Therefore, taking into account the complexity of enzyme inactivation, appropriate models must be used to represent these complex mechanisms.

In fact, Rojas; Trevilin and Augusto (2016) suggested that the enzymes can have several or even infinite intermediate states during processing, related with each spatial configuration associated with each value of the internal energy of the system. Each intermediate state can present the same, higher or lower activity as compared to the native state. As enzymes need energy (ε) to pass from one state (E) to another (eq. (4.12)), each energy quantum added to the system can be expected to result in a conformational change, which can change the enzyme activity. However, most works consider only one to four possibilities, which can be seen as a simplification (even though it can result in a good description of the experimental data).

$$E_0 \underset{\xleftarrow{\varepsilon_{(0)-(1)}}}{\rightleftharpoons} E_1 \underset{\xleftarrow{\varepsilon_{(1)-(2)}}}{\rightleftharpoons} \cdots \underset{\xleftarrow{\varepsilon_{(n-1)-(n)}}}{\rightleftharpoons} E_n \tag{4.12}$$

Therefore, the results in this work were evaluated by using the Weibull model (eq. (9)). This model was well applied in modelling enzyme (GINER et al., 2005; ELEZ-MARTÍNEZ; AGUILÓ-AGUAYO; MARTÍN-BELLOSO, 2006), microbial and spore inactivation, being considered a more flexible and realistic model (MAFART et al., 2002; VAN BOEKEL, 2002; ADEKUNTE et al., 2010). The Weibull model allows the possibility to represent the enzyme inactivation (SERMENT-MORENO et al., 2014)

considering not only one or two enzyme fractions, but infinite fractions (ROJAS; TREVILIN; AUGUSTO, 2016).

Particularly, the Weibull distribution model applied to survival data analysis considers that the individual organisms or molecules in a population do not have identical resistance and that their sensitivity to lethal agents (such as heat) is distributed. The inactivation times varies for each organism even when the population is pure, due to biological variation. In the case of enzymes, this is explained by the existence of isoenzymes and aggregates with different heat resistances. The inactivation curves can be considered as the cumulative form of a temporal distribution of lethal events; thus, the curves are reflections of different heat resistance distributions (PELEG; COLE, 1998). In other words, a lethal event is considered a probabilistic rather than a deterministic event (VAN BOEKEL, 2002).

The Weibull model was well fitted to the data obtained of POD inactivation for thermally treated samples with (US-TT) and without ultrasound pre-treatment (TT). The adjusted parameters and fit criteria are shown in Table 4.1. The POD inactivation data obtained along with the adjusted Weibull model prediction is presented in Figure 4.4 as a function of the equivalent processing time at 85 °C (reference temperature). The respective parity charts of the calculated residual activity and experimental residual activity are also shown in order to demonstrate the good fit. Note that the thermal inactivation of the samples pre-treated by ultrasound starts with $A_t/A_0 < 1$ (Figure 4B); this is due to the enzyme inactivation caused by the ultrasound pre-treatment (discussed in the previous section).

The coconut water POD showed high heat resistance. For example, at 80 °C, long times (>30 min) were necessary to reduce activity by 50%. Actually, the POD is considered a highly thermal resistant enzyme, used as an indicator of thermal process efficiency. The results are consistent with results reported by other authors that suggest long times and/or high temperatures to reduce the coconut water POD activity at an acceptable level (TAN et al., 2014). For instance, holding times of 310 s at 90 °C were needed for "complete" POD inactivation (i.e., activity reduction until undetected levels) in coconut water (CAMPOS et al., 1996). It is interesting to observe that a lower thermal resistance was reported when isolated commercial POD enzyme was added to sterilize coconut water (MATSUI et al., 2008) or added to coconut water simulated solutions (MATSUI et al., 2007), in comparison with the enzymes naturally present in the coconut water.
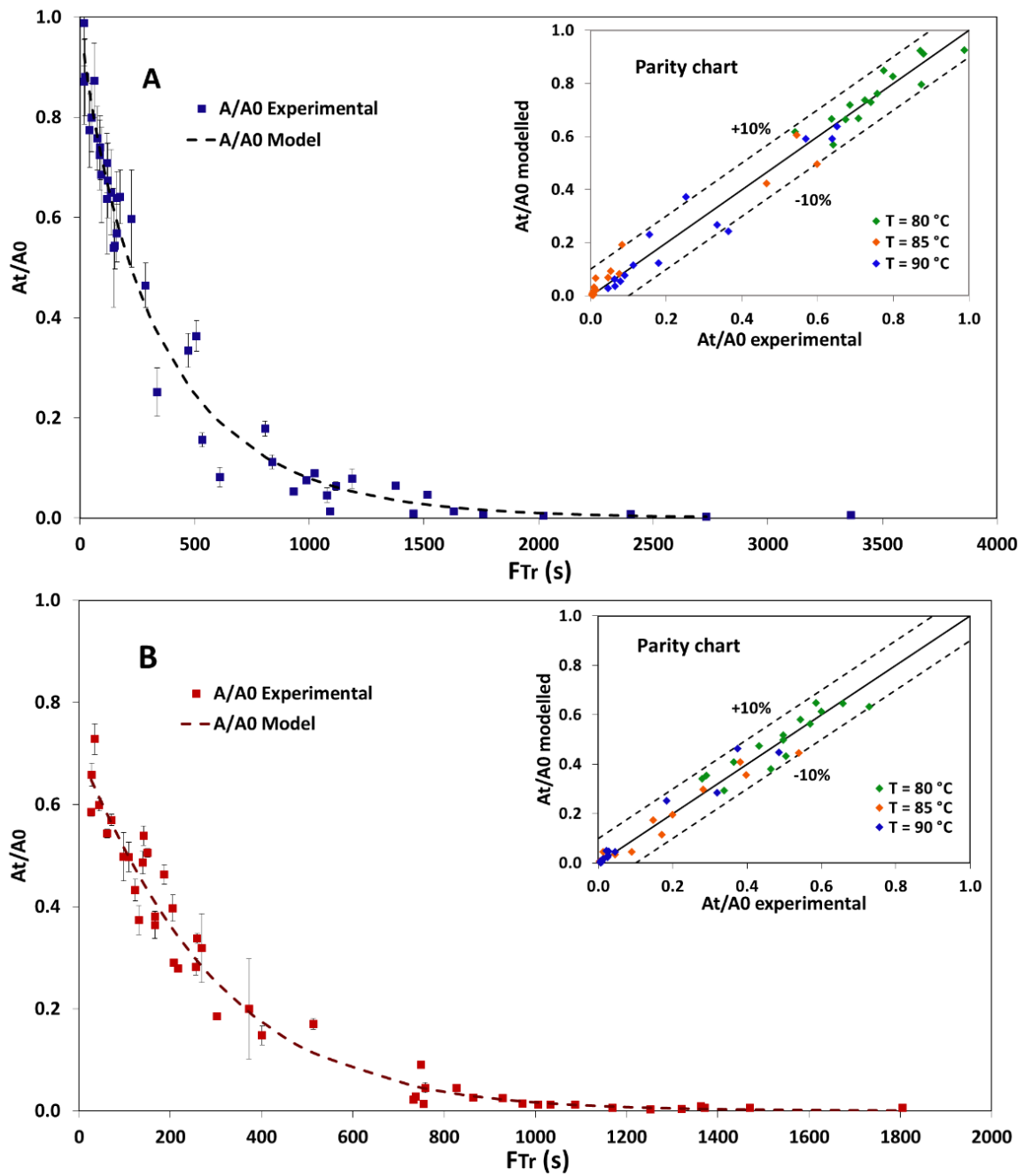
Figure 4.4 – POD residual activity (A_t/A_0) obtained along the thermal processing of coconut water without (A) and with US pre-treatment (B) as a function of the equivalent time (F_{T_r} - considering 85 °C as a reference temperature). The dots are the experimental values, the vertical bars are the standard deviation for each condition and the dashed curves are the adjusted Weibull model (eq. (9)). The respective parity charts between experimental and calculated values are also shown

On the other hand, with respect to the parameters obtained for the Weibull model (Table 4.1), the δ_{Tr} value was reduced by approximately 30% when the

ultrasound pre-treatment was applied. It indicates that the ultrasonic pre-treated coconut water needs less time to have the POD activity reduced in relation to the untreated product. In fact, by comparing a process to achieve 99% of POD inactivation (i.e., achieve A_t/A_0 = 0.01), and considering the temperature of 85 ºC, the US-TT sample requires approximately 46% less exposure time at this temperature than the TT samples. Moreover, the z value is observed to be slightly higher for the US-TT samples. For these samples, any increase in temperature has a smaller effect in the enzyme inactivation rate, i.e. the change in the δ_{Tr} value is reduced when compared with the TT samples.

Table 4.1 – Parameters adjusted and fit criteria of the Weibull model for inactivation of POD in coconut water for thermal treatment (TT) and thermal treatment after ultrasound (US-TT) data

Weibull parameters	Thermal processing (TT)	Thermal processing after ultrasound pre-treatment (US-TT)
$\delta_{(85\,°C)}$ (s)	896	640
z (°C)	4.51	5.09
β	0.86	1.08
λ	1.00	0.70
Fit criteria SSE	0.12	0.08
R² adjusted	0.97	0.97

Even though the Weibull model only gives a statistical account of a failure time distribution (WEIBULL, 1951), the value of the shape parameter (β) can be related with physiological effects (VAN BOEKEL, 2002). For microorganisms (PELEG; COLE, 1998; VAN BOEKEL, 2002), β < 1 indicates that the remaining cells have the ability to adapt to the applied stress and/or have a higher resistance, whereas β > 1 indicates that the remaining cells become increasingly damaged or have lower resistance. β = 1 means that each cell is equally susceptible no matter how long the treatment lasts, with the implicit assumption that there is no biological variation. These concepts can also be applied to the activity of enzymes.

According to López et al. (1994), the thermal inactivation of POD occurs either by dissociation or by degradation of the prosthetic group. Therefore, US probably also affects the enzyme structure, making the prosthetic group more exposed. Other possibilities of the US effect are dissociation of enzyme aggregates (LÓPEZ et al., 1994), changes in the active site three-dimensional structure (CRUZ; VIEIRA; SILVA, 2006) and molecular unfolding (FENG et al., 2016), among others. Consequently, the enzyme would be sensitized to heat presenting lower thermal resistance.

As observed in Table 4.1, the β value is less than one for the TT samples (β = 0.86), and approximately one for the US-TT samples (β = 1.08). Graphically, it means that the semi-logarithmic inactivation curves exhibit a concave shape for TT samples and a slight convex shape, almost linear, for US-TT samples. This variation in heat resistance in the TT samples could be explained by the existence of aggregates that increase the heat resistance of the enzyme (AUGUSTO et al., 2015). Such aggregates would be modified by the ultrasound, thus reducing their protective effect for thermal inactivation. The fact that the β value after US pre-treatment was approximately one indicates that the enzyme population has a higher homogeneity (i.e., fewer isozymes with different heat stability) after applying US technology. Therefore, a larger fraction of enzymes will share the same critical time of inactivation (which is a measure of resistance - Peleg and Shetty (1997) and Peleg and Cole (1998).

Regarding the λ value, it represents the initial activity reduction caused by the previous 30 min of US. The adjusted residual activity after US was λ = 0.70 in the adjusted model (Table 4.1), which is in good agreement with the experimental residual activity of 0.73 ± 0.07 after US. Figure 4.5 shows the POD residual activity only considering the effect of thermal processing, i.e., $A_t/\lambda A_0$ (where λ = 0.70 for the US-TT sample, and λ = 1.00 for the TT sample). This figure allows a comparison between the thermal inactivation of the samples with and without ultrasound pre-treatment, excluding the inactivation due to the ultrasound. Figure 4.5 shows the POD inactivation predictions from the Weibull model in semi-logarithmic scale for each process temperature (80, 85 and 90 °C) and also includes experimental data for comparison. The US-TT curves are verified to be below the TT curves; however, both are very close for short times, especially notorious at 85 °C, where the US-TT is almost linear and matches the TT curve for times under 200 s. Considering the assumption of several or infinite states (eq. (4.12)), the change of the shape to almost linear suggests that the

ultrasound pre-treatment largely reduced the number of states, showing minimum variation in thermal resistance among states, with the same susceptibility of being inactivated during the thermal processing.
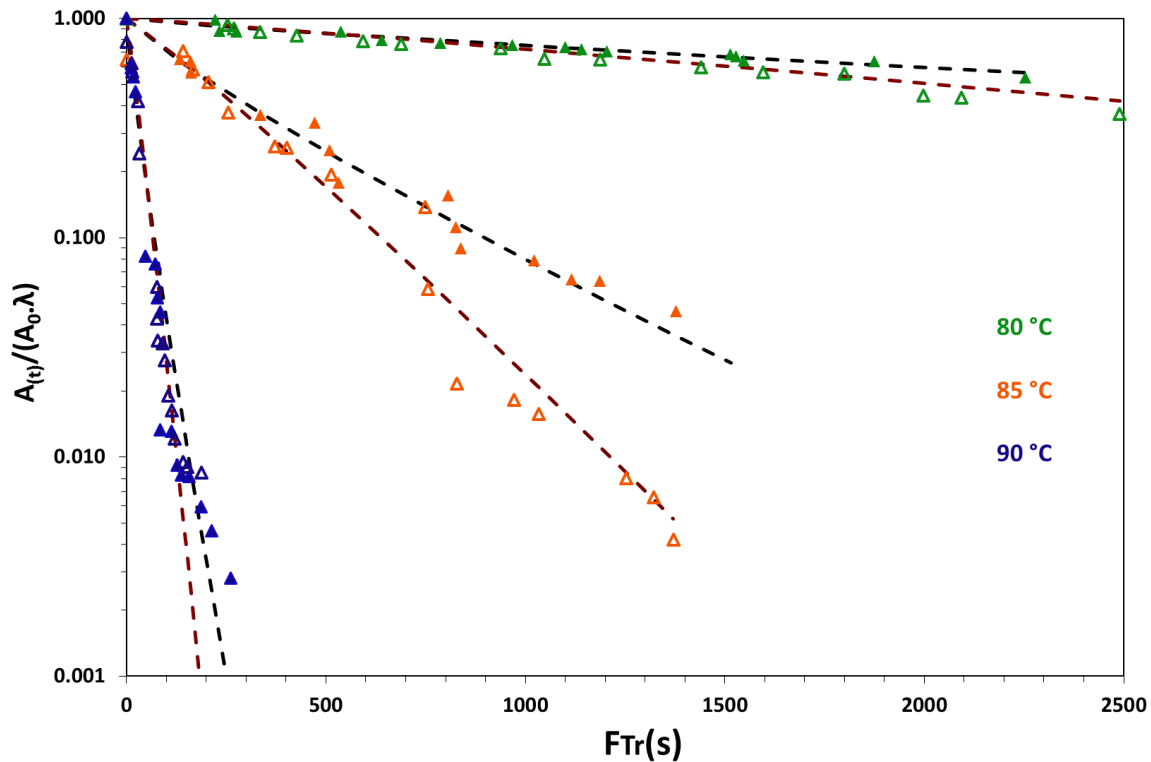

Figure 4.5 – POD inactivation without US pre-treatment (TT - filled triangles) and with US pre-treatment (US-TT - empty triangles) for the three evaluated temperatures
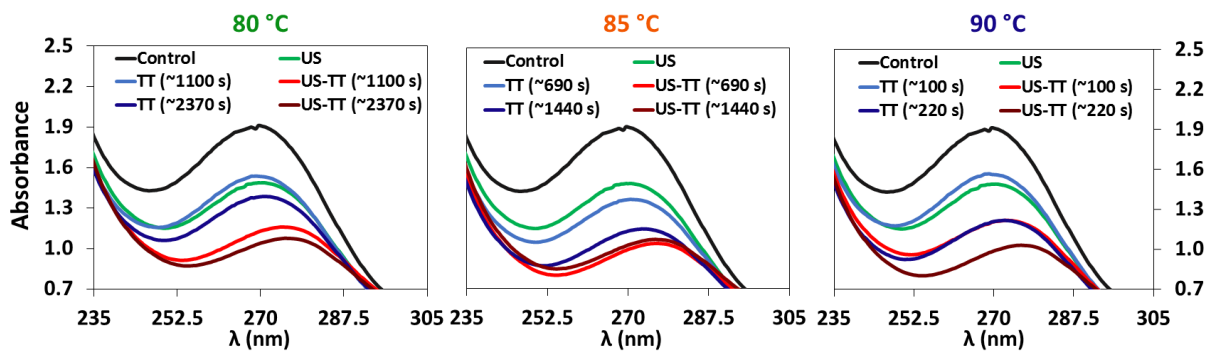

Figure 4.6 – Absorption spectra of the green coconut water without US pre-treatment (TT) and with US pre-treatment (US-TT) for the three evaluated temperatures at different time instants

Figure 4.6 shows the absorption spectra for the samples with and without US pre-treatment for all temperatures. It is observed that the absorption spectra of the pre-

treated samples (US-TT) are under the samples without the pre-treatment (TT) at the same time instant, due to the initial reduction caused by the ultrasound. The US-TT samples show less absorption variation during the thermal process time than the TT samples. For example, the absorbance at 270 nm was reduced by 16% and 0.5% for the TT and TT-US samples, respectively, from 690 s to 1440 s of thermal processing at 85 °C. These observations also contribute to the assumption of higher enzyme homogeneity after the ultrasound pre-treatment.

Therefore, it was described and demonstrated that ultrasound application in coconut water had positive results for promoting the thermal sensitization of POD (a thermal-resistant enzyme). This suggests the possibility of using US processing prior to conventional thermal processing, which could allow the use of lower temperatures and/or shorter times, with potential impacts on the consumer acceptance and on energy consumption.

4.4   Conclusions

Peroxidase (POD) is known to be a resistant enzyme responsible for undesirable effects on extracted green coconut water. This work demonstrated the effect of ultrasound (US) technology and its effectiveness for POD inactivation and thermal sensitization. US application alone reached levels of POD inactivation of approximately 27% after 30 min of processing at 286 W/L (20 kHz at 25 °C). Furthermore, US technology showed a positive impact on subsequent thermal processing. The enzyme thermal inactivation kinetics showed a non-linear behaviour, which was modelled according to the Weibull distribution function. The coconut water POD with ultrasonic pre-treatment showed smaller thermal resistance than the native one, due to the changes caused in the enzyme structure making the enzyme population more homogeneous. Therefore, its resistance distribution became more uniform, allowing smaller processing time and/or temperature and making the process easier to design and to evaluate. The results open new perspectives, allowing the use of milder thermal processing. Additionally, future works are needed to verify the product nutritional and sensorial qualities, which could potentially increase at lower processing time and/or lower temperatures. In fact, this work shows a potential application with both industrial and academic relevance.

Acknowledgement

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5 GENERAL CONCLUSIONS

The present work evaluated the ultrasound technology application in fruit beverages (peach juice and coconut water), as an alternative to improve the physical and enzymatic stability. It was demonstrated that the improved physical and enzyme stability is mainly due to the changes at structural level.

The ultrasound modified the structure of the peach juice particles and serum. It caused modification of intracellular structure and rupture of cell walls, consequently modifying the composition of the serum and the interparticle interaction. All these changes involved complex mechanisms, whose relative importance throughout the ultrasound processing varies. The interaction among them dictate the prevalence of one over another at each stage. Therefore, there was no predictable pattern for the evaluated properties throughout the process, especially the rheological properties and turbidity. The sedimentation stability was improved during the first minutes of processing and the colour stability was maintained along the storage.

The enzyme activity during ultrasound varies mainly depending on the characteristics of the ultrasound equipment used, the working conditions employed, raw material sources of the enzyme. The ultrasonic process can either increase, decrease or even do not change the enzyme activity. The ultrasound applied alone did not reach high levels of peroxidase inactivation. This is probably because more ultrasonic energy is needed to be inactivated, in other works was also proved that this enzyme is resistant to other processes as microwave or conventional thermal process. However, the ultrasound pre-treatment to thermal processing proved to be efficient in sensitizing the coconut water peroxidase – being this result interesting for further developments.

The coconut water POD inactivation during thermal processing was evaluated considering a non-isothermal process and enzyme inactivation kinetics was modelled using the Weibull distribution function adapted to this case. The non-isothermal evaluation, i.e., by considering the stage of heating and cooling as non-instantaneous, was carried out in order to a better approximation to the reality. Similarly, the best way to represent the enzyme inactivation is using primary models, more flexible and realistic, as the Weibull model. It was observed that the ultrasound pre-treatment modified the enzyme structure, making it more sensitive to the heat of the subsequent

thermal process. Further, the isozymes naturally present in coconut water remained structurally and catalytically more homogeneous after ultrasonic processing. Consequently, a larger fraction of enzymes shared the same time of inactivation during the thermal processing.

As a conclusion, the obtained results are useful from the academic point of view, because it involves a series of descriptions and technical explanation of the different effects produced by the ultrasound technology on both stability considering the physical properties and enzyme inactivation. On the other hand, the results also could have a positive impact from the industrial point of view, as it represents a new alternative for processing beverage, improving the physical and enzymatic stability without additives (stabilizers) or to prevent the use of severe temperatures and/or time with a negative impact on nutrients.

6 SUGGESTIONS FOR FUTURE RESEARCH

It is suggested for future research works, to study the effect of the ultrasound on the physical stability of different fruit juices, as probably the effects will be different from that described in this work, due to specificities of structure. It can also be studied the effect of ultrasound in a continuous process.

Additionally, it can be studied the ultrasound application in other types of enzymes, using model solutions with different composition, for a better evaluation and understand of its effect.

Further, the enzyme stability after ultrasound process and also ultrasound and thermal processing, can also be studied over storage, evaluating the possible activity recovery.

APPENDIX

Appendix A – Simple abstract

English

There is an increasing demand for food beverages with fewer additives, good visual, sensory and nutritional attributes. In this work, the application of the ultrasound technology (US) for beverage processing was studied in order to improve stability and physical properties of peach juice and green coconut water. In fruit juice with pulp (peach juice), the US improved the stability (i.e., it was observed a homogeneous juice without separation in layers), consistency and colour retention over storage. When the coconut water was processed whit US, depending on the energy applied to the samples, an increase, decrease or even any modification on enzyme activity was obtained. In addition, US was used as a pre-treatment prior to thermal processing of coconut water. The enzymes become more sensitive to thermal processing when previously processed with US. Therefore, the US application improved the stability without the need for stabilizing additives, as well as promoted the enzyme inactivation, decreasing the severity of the conventional thermal processing.

Keywords: Food Engineering, Ultrasound Technology; Thermal process; Fruit beverages, Physical Properties; Enzyme inactivation

Português

A demanda por bebidas tem aumentado, especialmente aquelas com menor quantidade de aditivos, bons atributos visuais, sensoriais e nutricionais. Neste trabalho, a aplicação da tecnologia de ultrassom (US) foi estudada no processamento de bebidas, com o objetivo de melhorar a estabilidade e propriedades físicas em suco de pêssego e água de coco verde. No suco de fruta com polpa (suco de pêssego), o US melhorou a estabilidade (i.e., observou-se suco homogêneo, sem separação em camadas), consistência e retenção de cor ao longo do armazenamento. Quando a água de coco foi processada com US, dependendo da energia aplicada às amostras, houve aumento, diminuição ou mesmo nenhuma modificação na atividade enzimática. Além disso, o US foi utilizado como pré-tratamento do processamento térmico de água de coco, tornando as enzimas mais sensíveis ao processamento. Portanto, a aplicação do US melhorou a estabilidade sem a necessidade de aditivos estabilizantes, assim como também promoveu a inativação de enzimas, diminuindo a severidade do processamento térmico convencional.

Palavras-chave: Engenharia de alimentos, Tecnologia de ultrassom; Processamento térmico; Bebidas de frutas, Propriedades físicas; inativação enzimática

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Español

Actualmente existe una creciente demanda de bebidas con menos aditivos, buenos atributos visuales, sensoriales y nutricionales. En el presente trabajo, se estudió la aplicación de la tecnología de ultrasonido (US) en el procesamiento de bebidas, con el objetivo de mejorar la estabilidad y propiedades físicas en jugo de durazno y agua de coco verde. En jugo de fruta con presencia de pulpa (jugo de durazno), se obtuvo mejora de la estabilidad (es decir se observa un producto homogéneo sin separación de fases), consistencia y conservación del color a lo largo del almacenamiento. Cuando se aplicó US en el procesamiento de agua de coco, dependiendo de la energía aplicada a las muestras, fue obtenido un incremento, disminución o incluso ninguna modificación en la actividad enzimática. Además, se usó US como pretratamiento antes del proceso térmico de agua de coco. Las enzimas se volvieron más sensibles al procesamiento térmico cuando fueron previamente procesadas con US. Por lo tanto, la aplicación de US mejoró la estabilidad sin la necesidad de usar aditivos estabilizantes, además de promover la inactivación enzimática, disminuyendo la severidad del proceso térmico convencional.

Palabras clave: Ingeniería de alimentos, Tecnología de ultrasonido; Proceso térmico; Bebidas de frutas, Propiedades físicas; Inactivación enzimática

Appendix B - Experimental images

Equipment used for peach juice and coconut water processing. Left: Ultrasound probe, Right: Ultrasound bath

Left: adaptation of thermocouples in the samples. Right: Thermal processing in thermostatic bath with stirring

Pulp sedimentation after 21 days of storage at 25 °C. US-0min, US-3min, US-6min, US-10min and US-15 min (from left to right, respectively)

Turbidity of the supernatant in peach juice samples after centrifugation. Left: sample without ultrasonic processing. Right: Sample with 15 min of ultrasonic processing

Appendix C - First page of the published chapter 2

Peach juice processed by the ultrasound technology: Changes in its microstructure improve its physical properties and stability

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ABSTRACT

Ultrasound is a non-conventional processing technology, which can be used not only for food preservation, but also to improve its properties and quality. This study evaluated the physical properties and stability of peach juice processed by the ultrasound technology. The peach juice processed by the ultrasound technology showed changes in its structure, evidenced by the optical microscopy and particle size distribution, involved steps of cell damage and release of intracellular content, particle size reduction, disruption of the whole cells, polysaccharide size reduction and dispersion of constituents. These effects, depending of the processing time, can trigger different mechanisms with a complex behaviour. The interaction among them and the relative importance of each one change during processing, determining the final rheological properties, pulp sedimentation and serum cloudiness (turbidity). The results indicated that the ultrasound technology can be used to improve the physical properties of peach juice, increasing the stability to pulp sedimentation and serum cloudiness, maintaining or increasing the juice consistency, with insignificant colour changes during the storage.

1. Introduction

Ultrasound (US) is defined as sound waves having frequency that exceeds the limit of the human ear (~20 kHz). This technology has been used as alternative to conventional food processing. Based on the frequency range, the applications of ultrasound in food analysis, quality control and processing can be divided into low energy (frequencies higher than 100 kHz at intensities below 1 W·cm^{-2},) and high energy (intensities higher than 1 W·cm^{-2} at frequencies between 20 and 500 kHz) (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). The use of power ultrasound (high energy) in processing, which due the acoustic and hydrodynamic cavitations are able to induce chemical and physical changes in different food systems (Awad et al., 2012; Chemat, Zill e, & Khan, 2011). In fluid food systems, such as juices, subjected to sonication, a number of physical and mechanical effects can result. Large particles of a liquid suspension are subject to surface erosion (by cavitation collapse in the surrounding liquid) or particle size reduction (due to fission through interparticle collision or the collapse of cavitation bubbles formed on the surface) (Mason, Paniwnyk, & Lorimer, 1996). Several studies have evaluated the use of the US technology as alternative for fluid food processing facilitating operations such as emulsification (Gaikwad & Pandit, 2008), modifying the functional properties of different food proteins (Yanjun et al., 2014)

and assisted the extraction of various food and bioactive compounds (Barba, Brianceau, Turk, Boussetta, & Vorobiev, 2015; Roselló-Soto et al., 2015).

In fact, many works were carried out relating the processing of fruit juices using the US technology as alternative for total or partial substitution of thermal processing (Zinoviadou et al., 2015). Thermosonication with low temperature could enhance the inactivation of enzymes and microorganisms and it was used as a potential preservation technique in pear juice (Saeeduddin et al., 2015), tomato juice (Ertugay & Başlar, 2014; Terefe et al., 2009; Wu, Gamage, Vilkhu, Simons, & Mawson, 2008), watermelon juice (Rawson et al., 2011), orange juice (Walkling-Ribeiro, Noci, Cronin, Lyng, & Morgan, 2009), apple juice (Abid et al., 2014; Baslar & Ertugay, 2013), grapefruit juice (Aadil et al., 2015), among others.

At present, the effect of US technology on the physical properties of food products such as the colour degradation and quality parameters in orange juice (Tiwari, Muthukumarappan, O'Donnell, & Cullen, 2008; Tiwari, O' Donnell, Muthukumarappan, & Cullen, 2009), cloudy quality of apple juice (Ertugay & Başlar, 2014), colour stability and apparent viscosity of pineapple juice (Costa et al., 2013), colour stability, solid deposition and apparent viscosity of cactus pear (Cruz-Cansino et al., 2015), colour and sensory quality (appearance, texture, taste and aroma) of soursop juice (Dias et al., 2015), has been studied. These previous works indicated that this technology could be used to increase and improve the properties of juices such as the consistency (apparent viscosity), colour, cloudy stability and its sensory acceptance.

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Appendix D – First page of the published chapter 3

The ultrasound technology for modifying enzyme activity

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Abstract

Enzymes are protein complexes compounds widely studied and used due to their ability to catalyze reactions. The food processing mainly aims the inactivation of enzymes due to various undesirable effects. However, there are many processes that can be optimized by its catalytic activity. In this context, different technologies have been applied both to inactivate or to improve the enzymes efficiency. The Ultrasound technology emerges as an alternative mainly applied to achieve the enzyme inactivation. On the contrary, very few investigations show the ability of this technology under certain conditions to achieve the opposite effect (i.e. increase the catalytic activity of enzymes). The objective of this study was to correlate the ultrasonic energy delivered to the sample (J/mL) with the residual enzymatic activity and explain the possible mechanisms which results in the enzymatic activation/inactivation complex behavior. The activity of POD in coconut water was evaluated as a model. The enzymatic activity initially increased, followed by reduction with a trend to enzyme inactivation. This complex behavior is directly related to the applied ultrasonic energy and their direct mechanical effects on the product, as well as the effect in the enzymatic infinite intermediate states and its structural conformation changes. The obtained results are useful for both academic and industrial perspectives.

Keywords: peroxidase(POD), enzymatic activity, enzymatic structural conformation, ultrasound technology, coconut water.

1. Introduction

The enzyme-catalysed reactions are important in pharmaceutical, chemical, non-alimentary and alimentary industry. It is important during the processing and preservation of food, since the activity of enzymes includes undesirable reactions such as browning, rancidity, discoloration, loss of texture, among others. In this case, it is necessary to inactivate the enzymes. However, there are enzymes that catalyse desirable reactions, such as that used for hydrolysis, clarifying or to soften meat. Traditional thermal methods such as sterilization, pasteurization, precooking or blanching are the methods most known and used by the food industry for the inactivation of enzymes, although many new technologies are also studied and applied. Biotechnological techniques and also some innovative technologies are also used to improve and increase the enzymatic efficiency. In fact, the ultrasound technology is gaining importance. There are many studies of ultrasound application to achieve enzyme inactivation, such as for lipases, proteases, peroxidase (POD), polyphenol-oxidase (PPO), polygalacturonase (PG), pectinesterase, pectinmethylesterase (PME), ascorbate peroxidase (APx) (Costa *et al.*, 2013; Huang *et al.*, 2015; Tiwari *et al.*, 2009; Vercet *et al.*, 2001). On the other hand, few works demonstrate the capacity of these technology to enhance the enzyme activities.

It is difficult to identify the specific enzyme mechanism during sonication, which could be due to a singular or combination of several chemical and physical effects occurring simultaneously (Rawson *et al.*, 2011).

In this work, the coconut water is

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