Ultrasound–assisted Liquefaction of Honey

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Ultrasound-assisted Liquefaction of Honey

PhD Thesis submitted to obtain the degree of Doctor from the Universitat Politècnica de Catalunya

by

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This work has been carried out under the direction of Dr. Francesc Sepulcre Sánchez.

Signature:

Dr. Francesc Sepulcre Sánchez

I lovingly dedicate this Thesis to the memory of my father, Dr Nader Kabbani, who made

me believe in the richness of learning.

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Abstract

Ultrasound-assisted Liquefaction of Honey

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Crystallization of honey is a common process of the honey industry. Liquid honey is preferred by most of the consumers and by food companies for ease of handling. Honey is commonly heated during pasteurization in order to liquefy it and inhibit any microbial growth. However, heating can degrade the main quality parameters of honey.

A better method compared to expensive and time-consuming heating is desirable to pasteurize, accelerate the liquefaction and retard the crystallization process in honey.

The present thesis documents the work done at investigating the effect of the ultrasounds (US) in honey liquefaction, quality alteration and honey decontamination.

Firstly, in Chapter 1, the effect of different combinations of US treatment (power, temperature and duration) on honey liquefaction were evaluated by studying the rheological properties of honey; viscosity behaviour, crystal content, tendency to re-crystallization and thermal properties. Secondly, in Chapter 2, the effects of US on the hydroxymethylfurfural concentration and diastase activities in honey were determined by chemical analysis and compared with that for standard heat-treated honey samples. Thirdly, in Chapter 3, US treatment was investigated for honey decontamination. In addition, the *in vitro* antimicrobial and antifungal activities of ultrasonicated honey against several types of microorganisms were evaluated.

The results obtained in this research point to a successful application of the ultrasound technology for the liquefaction of honey, as it speeds up its liquefaction, do not degrade the quality and the intrinsic biological activity of honey was neither affected.

Resumen

Liquefacción de la mel asistida por ultrasonidos

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La cristalización de la miel es un proceso común de la industria de la miel. La miel líquida es preferida por la mayoría de los consumidores y por las compañías procesadoras por mayor facilidad en su manejo. La miel se calienta comúnmente durante la pasteurización con el fin de licuarla e inhibir cualquier crecimiento microbiano. Sin embargo, el calentamiento que sufre puede degradar su calidad.

Un método mejor, en cuanto a coste y tiempo de procesamiento en comparación con el método de calentamiento actual, es deseable para pasteurizar, acelerar la licuefacción y retardar el proceso de cristalización en la miel.

La presente Tesis documenta el trabajo realizado en la investigación del efecto de un tratamiento de ultrasonidos (US) en la liquefacción, calidad y descontaminación de la miel.

En el primer Capítulo, el efecto de diferentes combinaciones de tratamiento de US (potencia, temperatura y duración) para la licuefacción de la miel, se evaluaron mediante el estudio de las propiedades reológicas; comportamiento de la viscosidad, contenido de cristales, tendencia a re-cristalización y propiedades térmicas. En el segundo Capítulo, los parámetros de calildad, contenido de hidroximetilfurfural y actividad diastásica de la miel, se determinaron por análisis químico. En el tercer Capítulo, se investigó el efecto de los US en la descontaminación de la miel, así como sus propiedades antimicrobianas.

Los resultados obtenidos en esta investigación apuntan a una aplicación exitosa de la tecnología de ultrasonido para licuar la miel, ya que acelera la licuefacción, no degrada la calidad y la actividad antimicrobiana intrínseca de la miel no se ve afectada.

Resum

Liquefacció de la mel assistida per ultrasons

Dania Kabbani Rahima

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Director: Dr. Francesc Sepulcre

La cristal · lització de la mel és un procés comú de la indústria de la mel. La mel líquida és preferida per la majoria dels consumidors i per les companyies processadores per a major facilitat en el seu maneig. La mel s'escalfa comunament durant la pasteurització per tal de liquar-la i inhibir qualsevol creixement microbià. No obstant, el calentament pot degradar els seus principals paràmetres de qualitat.

Un mètode millor, en comparació amb l'escalfament actual, car i que consumeix molt de temps, és desitjable per pasteuritzar, accelerar la liqüefacció i retardar el procés de cristal.lització en la mel.

La present Tesi documenta el treball realitzat en la investigació de l'efecte d'un tractament d'ultrasons (US) en la liquefacció, qualitat i descontaminació de la mel.

En el primer Capítol, l'efecte de diferents combinacions de tractament d'US (potència, temperatura i temps) per a la liqüefacció de la mel, es van avaluar mitjançant l'estudi de les propietats reològiques, comportament de la viscositat, contingut de cristalls, tendència a re-cristal·lització i propietats tèrmiques. En el segon Capítol, els paràmetres de qualiltat aixi com a contingut d'hidroximetilfurfural i activitat diastàsica, es van determinar per anàlisi química. Al tercer Capítol, es va investigar l'efecte dels US en la descontaminació de la mel, així com les seves propietats antimicrobianes.

Els resultats obtinguts en aquesta investigació apunten a una aplicació reeixida dels ultrasons, ja que acceleren la liqüefacció, no degraden la qualitat i l'activitat antimicrobiana intrínseca de la mel no es veu afectada.

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1. General Introduction

Honey is the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (Codex 1998, EC 2001/110).

There is **growing market interest in honey** as a natural sweetener with multiple nutritional and functional benefits. The worldwide productions in 2011 were 1,633699 tonnes of honey. The European Union (EU) is an important producer of honey, accounting for around 13 % (217,366 tonnes in 2010) of the global honey production. **Spain is also an important producer in Europe**, with nearly 34,000 tonnes of honey produced in 2011 (FAOSTAT, 2013). In general, the honey market is a very stable market. Nevertheless, it is still evolving. EU imports of honey amounted to 375 million € in 2007. Germany, the UK, Spain, Italy, Belgium and France were the largest EU markets for imports of honey from developing countries. In 2007, apparent honey consumption in the EU amounted to 310 thousand tonnes, with expectations of further increasing (CBI, 2009).

Honey is known for its effectiveness in instantly boosting the performance, endurance and reduces muscle fatigue of athletes (N. Altman, 2010). Furthermore, recent claims about the health benefits are associated with honey consumption, such as wound healing, restorative sleep, cough suppression and cognitive function. Honey has been useful in the treatment of infected surgical wounds, burn wounds, and decubitus ulcers (bedsores) (D. Mohapatra *et al.* 2010). In addition, it is often used for treating digestive problems such as diarrhea, indigestion, stomach ulcers and gastroenteritis (C. Voidarou *et al.* 2010). Other recent scientific reports linking honey to health include research suggesting that taking honey in combination with calcium supplements could help boost bone strength (R. Elkins, 1996). Research such as these has helped boost honey consumption around the world, with sales increasing 14 per cent between 2003 and 2011 (FAOSTAT, 2013).

At a biochemical level, honey is a natural supersaturated sugar solution mainly composed of a complex mixture of carbohydrates. Fructose and glucose account for nearly 60-85 % as predominant monosaccharides, maltose and sucrose as the most important disaccharides, and other low molecular weight oligosaccharides. There are also other minor constituents such as proteins, enzymes (mainly invertase, glucose oxidase, catalase and phosphatases), amino and phenolic acids, flavonoids and minerals. The composition of honey depends on the plant species pollinated by the honeybees and the climatic, processing and storage conditions (J. White, 1975; B. d'Arcy, 2007; S. Ajlouni and P. Sujirapinyokul, 2009; S. Saxena, S. Gautam and A. Sharma, 2009).

The composition of honey (sugars and moisture content) is responsible for many of the physicochemical properties of honey, such as viscosity, hydroscopicity, and granulation. Water content as well as the water activity (a_w) are the major factors that influence the keeping quality or storability of honey (E. Tosi *et al.*, 2004). The moisture content of honey ranges from 16 %-18 % and the water activity is commonly 0.6. The low a_w and high osmotic environment do not support microbial growth, and hence, honey is self stable. Recently harvested raw honey is in a liquid state, but it crystallizes with greater or lesser speed depending on numerous factors such as origin (botanical and geographical), temperature, moisture content, and sugar content. Since **honey is a supersaturated solution of glucose, it has a tendency to crystallize spontaneously at room temperature in the form of glucose monohydrate**. Crystallization of honey, commonly called granulation or crystallization, is **an undesirable process in liquid honey because it affects the textural properties**, making it less appealing to the consumer, who prefers it liquid, and/or transparent (E. Tosi *et al.*, 2004; S. Ajlouni *et al.*, 2009; S. Saxena *et al.*, 2009; I. Escherique 2009).

Crystallization poses a major problem in the liquid honey industry. On the one hand, crystallization is an undesirable property in the handling and processing of honey, as it substantially limits the flow of unprocessed honey out of the storage containers and through the processing facilities, thereby reducing throughput times and yields, which has negative financial implications for processors. On the other hand, appearance plays a key role in the commercial success of honey. Consumers demand a fluid, non-crystallized product (I. Eschrique, 2009). Hence, in terms of consumer appeal, crystallised honey is generally regarded as unacceptable.

To slow down the natural crystallization process and ensure stability during its commercial life, raw honey is normally pasteurized prior to being packaged. **Honey is heated** to a temperature of 57 °C for one hour or 60 °C for 30 min or above in the packing plant to kill the yeast, and thus inhibit fermentation. Flash heating honey up to 70 °C or even up to 77 °C for 5 min is the commercial practice of pasteurisation (G.F. Townsend, 1976; E. Crane, 1990, B. d'Arcy 2007). However**, heating has been shown to have an effect on honey quality parameters**; it may destroy bionutrients, and produce a simultaneous decrease in diastase activity and an increase in hidroxymethylfurfural (HMF) content (E. Tosi 2002, B. d'Arcy 2007). In fact, these two parameters are used as controls so as to limit thermal treatment.

Due to the limitations of heat treatment, there is a **real need to provide honey producers with a non-thermal** alternative for controlling the crystallization of honey, thereby facilitating processing and flow in the packaging plants, as well as meeting with consumer demand, for high quality, nutritionally beneficial clear honey that has not been damaged by heat treatments. Therefore, the honey industry requires alternate technologies for liquefying candied honey, such as ultrasound.

High-power low-frequency **ultrasounds have become an alternative to many conventional food-processing steps**, such as homogenization, milling, high shear mixing, pasteurization and solid/liquid separation. Moreover, it has shown to improve the efficiency of traditional processes such as filtration/screening, extraction, crystallization and fermentation. In honey, it is believed that sonication destroys the yeast, improves the appearance and inhibits the granulation of the product.

2. Thesis Objectives

The aim of this thesis is the study of the incidence of an ultrasound treatment on naturally crystallised rosemary honey. To this end, the study will be divided into three chapters;

First, in **Chapter 1**, the effect of different combinations of ultrasound treatment (power, temperature and duration) on honey liquefaction will be evaluated by studying the rheological properties of honey; viscosity behaviour, crystal content, tendency to re-crystallization and thermal properties.

Secondly, in **Chapter 2**, the effect of ultrasounds on the main quality parameters of hydroxymethylfurfural concentration and diastase activity in honey will be determined when exposed to an ultrasound treatment process for liquefying crystallized honey and compared with that for standard heat-treated honey samples. Sugar composition will be also analysed to determine whether a change of composition occurs.

Thirdly, in **Chapter 3**, ultrasound treatment will be also evaluated for honey decontamination. On the other hand, the *in vitro* antimicrobial and antifungal activities of ultrasonicated honey against several types of microorganisms will be evaluated.

3. Literature Review

3.1. Honey composition

Each honey is as unique as the flowers it came from, as different as the geographies the bees roam and forage, and as complicated as a blend of a thousand perfumes. *Every hive produces its own secret blend* (K. Flottum, 2009).

Rosemary (*Rosmarinus officinalis*) is an evergreen aromatic bush, which reaches a height of about 2 m with cylindrical leaves of about 4 cm long. Flowers are grouped in bunches on the leaves. The calyx is a red or green colour, divided in two lips covered in hairs. The corolla is a pale blue colour made up of two lips, the bottom one is where the insects alight and another smaller one on top. It generally blooms in the Spring and Autumn. The origin of this honey is located in the Central and Central Eastern Spain, mainly in the Valencian Community, Catalonia, Andalucía, Extremadura, Aragón, Murcia and Castilla-La Mancha. Spanish Rosemary honey is taken from the mountain sides of Spain where the wild flora is far from the drift of chemical sprays.

Picture 1**. Rosemary Honey**

Rosemary honey has a creamy and dense texture, characterized by fine crystals. Its colour is clear with a floral perfume balance and good feeling aromas, highlighting its wild scent of rosemary flowers and a delicate flavour. Rosemary honey has delicate aroma, slightly balsamic, not too intense but perceptible. Generally has sweet taste. The main therapeutic applications are for rheumatism, gout, cirrhosis, as a tonic for the stomach and respiratory ailments.

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The overall composition of honey is shown in Table 1. The carbohydrates are the main constituents, comprising about 95 % of the honey dry weight. Beyond carbohydrates, honey contains numerous compounds such as organic acids, proteins, amino acids, minerals, polyphenols, vitamins and aroma compounds. It should be noted that the composition of honey depends greatly on the botanical origin, a fact that has been seldom considered in the nutritional and physiological studies.

Table 1. Honey composition (data in g/100 g).

	Blossom Honey	
	average	min. - max.
Water	17.2	15 - 20
Monosaccharides		
Fructose	38.2	30 - 45
Glucose	31.3	24 - 40
Disaccaharides		
Sucrose	0.7	0.1 - 4.8
Others	5.0	2.0 – 8
Trisaccharides		
Melezitose	<0.1	
Erlose	0.8	0.5 – 6
Others	0.5	0.5 – 1
Undetermined oligosaccharides	3.1	
Total sugars	79.7	
Minerals	0.2	0.1 - 0.5
Amino Acids, Proteins	0.3	0.2 - 0.4
Acids	0.5	0.2 - 0.8
pH-value	3.9	3.5 - 4.5

Data extracted from S. Bognadov (2009)

3.1.1. HONEY CARBOHYDRATES

Monosaccharides, disaccharides and trisaccharides

Fructose and glucose are monosaccharides that account for about 85-95% of honey carbohydrates (E. Crane, 1990; B. d'Arcy, 2007). Besides, about 25 different sugars have been detected (S. Bogdanov, 2002). Honeybees adds the enzyme invertase to the nectar or honeydew, which produces fructose and glucose by hydrolysis of the sucrose present in the nectar. The fructose to glucose ratio is a characteristic of some honeys. Most honey types including robina, salvia, tupelo and sweet chestnut honeys are richer in fructose than glucose. However, canola, dandelion and blue curls honeys are unusually richer in glucose (D. M. Amor, 1978).

Sucrose is a disaccharide that comprises 1 – 3 % of the composition of honey, and consists of fructose and glucose units linked together by a glycosidic bond. J. W. White and N.Hoban (1959) isolated and identified isomaltose, maltulose, turanose and nigerose from honey. They also confirmed the presence of sucrose and maltose in honey. Spanish honeys contain sucrose, maltose, and kojibiose (R. Mateo and F. Bosch-Reig, 1997). Da Costa Leite *et al.* (2000) reported that Brazilian honey contains sucrose, maltose, turanose, nigerose, melibiose and isomaltose. B. Mossel *et al.* (2002) reported concentrations of sucrose maltose and turanose (0.37 – 1.44 g/100 g honey) in 126 samples of honeys from 15 different species-specific types of Australian honey (cited by B. d'Arcy, 2007).

Trisaccharides consist of three simple sugar subunits and oligosaccharides contain more than three sugar units. The presence of erlose was first reported by J. W.White and J. Maher (1953). R. Mateo and F. Bosch-Reig (1997) showed that Spanish honey contains maltulose, raffinose, erlose and melezitose. Da Costa Leite *et al.* (2000) analysed 70 Brazilian honeys of different floral types and confirmed the presence of maltotriose, panose, melezitose and raffinose.

3.1.2. PROTEINS, ENZYMES AND AMINO ACIDS IN HONEY

The amounts of amino acids and proteins are relatively small, at the most 0.7 %, thus having relatively small nutritive effects (S. Bogdanov, 2002). However these components can be important for judging the honey quality. Honey contains a wide variety of essential amino acids (J. Cotte *et al.,* 2004). The amino acid proline, which is added by bees, is a measure of honey ripeness (W. Von der Ohe, 1994). The proline content of normal honeys should be more than 200 mg/kg. Values below 180 mg/kg mean that the honey is probably adulterated. About other 26 free

amino acids are known to occur in honey, but they are present in small amounts with little nutritional significance (I. Hermosín *et al.,* 2003). These other amino acids do not play a key role for the determination of quality or origin of honey. In a study carried out with 92 samples of honeys from 17 botanical and 4 geoFigureal different sources (L. S. Conte *et al.*, 1998) amino acids were isolated and, after derivatization, analysed by gas chromatography. For rosemary honey, tyrosine was the main amino acid, and also high amounts of proline and phenylalanine. I. Hermosín *et al.,* (2003) carried out a study with 48 samples honeys, from six different regions of Spain and 10 botanical sources. The authors reported the rosemary honey type (11 samples were analysed) contained 23 types of amino acids, being phenylalanine (28.4 ± 32.7 mg/100 g of honey), proline (28.0 ± 14.8 mg/100 g of honey) and tyrosine (11.2 ± 9.4 mg/100 g of honey) the most prevalent amino acids. However, it is important to mention that the high standard deviation values are because the amino acid concentration varies greatly from one sample to another, even being from the same botanical origin.

The honey proteins are mainly enzymes, reviewed by J. W. White (1975). Enzymes are another important constituent of honey because they play an important role in honey production from the nectar of the plant (S. Bogdanov, 2002). Further, enzymes are heat sensitive and extra low levels indicate that honey has been overheated. Further, their activities are decreased during storage and used as indicators of the freshness of honey (B. d'Arcy, 2007). The three main honey enzymes are diastase (amylase, EC.3.2.1.1), decomposing starch or glycogen into smaller sugar units, invertase (sucrase, α-glucosidase, EC. 3.2.1.26), decomposing sucrose into fructose and glucose, as well as glucose oxidase (EC.1.1.3.4), producing hydrogen peroxide and gluconic acid from glucose.

Honeybees add invertase to nectar during the process of harvesting and ripening of honey. The hydrolytic activity of invertase on sucrose finally produces glucose and fructose. Invertase is more susceptible to damage by storage and heat and is used in some countries as an indicator for honey virginity and freshness. Fresh and virgin honeys are supposed to have at least 10 Hadorn invertase units (SN). Invertase activity is expressed as g sucrose hydrolysed per 100 g/h (SN).

J. S. Bonvehi *et al.,* (2000) studied the invertase activity of Spanish monofloral honeys from different floral origins and reported that invertase activity of rosemary honey type was 14.43 ± 5.73 SN.

Glucose oxidase is active only in unripe or dilute honey, and most active when the sugar concentration is between 25 % and 30 % (B. d'Arcy, 2007). This enzyme oxidises glucose and produces gluconic acid and hydrogen peroxide. Hydrogen peroxide is the major contributor to the antimicrobial activity of honey, and the different concentrations of this compound in different honeys result in their varying antimicrobial effects (P. C. Molan, 1992). The activity of glucose oxidase decreases with an increase in reducing sugar concentration during honey production, and disappears when nectar is fully converted to reducing sugars (B. d'Arcy, 2007).

Diastase (and invertase) play an important role for judging of honey quality and are used as indicators of honey freshness. A minimum value of 10 diastase units is set in the Codex Alimentarius (Codex Stan for Honey) and the European honey directive (EC 2001/11). Their activity decay upon storage and heating of honey. However, the diastase and invertase activity vary in wide limits depending on the botanical origin of honey and thus have a limited freshness indicating power. HMF is the better quality criterion in this respect (see 1.4. Section. HMF content of honey).

3.1.3. VITAMINS, MINERALS AND TRACE COMPOUNDS IN HONEY
Honey is rather low in vitamin content. The main vitamins found in honey are thiamin (B1), riboflavin (B2), pyridoxin (B6), phyllochinon (K), niacin, panthothenic acid and ascorbic acid (C).

Minerals are found in the ash component of honey. J. W. White (1978) reported an average ash content of 0.17 %, with a range of 0.02-1.03 % in honey. The minerals present in honey are potassium, sodium, calcium, magnesium, iron, copper, manganese, chlorine, phosphorus, sulphur and silica. Potassium is the major element, which on average makes up 33-35% of honey ash, while iron, copper and manganese are present in honey in small amounts. The mineral elements of honey published by J. W. White (1978) are given in Table 2.

Honey contains a number of other trace elements. From the alimentary point of view the minerals chrome, manganese and selenium are of nutritional importance (S. Bogdanov, 2002). The elements sulphur, boron, cobalt, fluorine, iodine, molybdenum and silicon can be important in human nutrition too.

Honey contains 0.3-25.0 mg/kg choline and 0.06 to 5.00 mg/kg acetylcholine. Choline is an essential for cardiovascular and brain function, and for cellular membrane composition and repair, while acetylcholine acts as a neurotransmitter (S. Bogdanov, 2002).

Table 2. Mineral elements detected in honey

Mineral Element	Honey Colour	As percentage of ash	
		Range	Average
Potassium	Light	23.0-70.8	35.30
	Dark	2.0-61.6	33.00
Sodium	Light	0.96-9.26	3.59
	Dark	0.2-11.20	4.68
Calcium	Light	3.54-13.00	8.77
	Dark	0.46-7.30	3.57
Calcium as lime (CaO)	Light	4.95-18.19	12.27
	Dark	0.64-10.21	5.00
Magnesium	Light	1.00-9.24	3.42
	Dark	0.66-11.47	2.77
Chlorine	Light	4.52-13.21	10.20
	Dark	2.26-14.46	9.67
Phosporus	Light	1.03-9.55	6.37
	Dark	0.84-6.67	3.67
Sulphur	Light	5.77-16.24	11.49
	Dark	2.67-14.36	7.98
Silica	Light	0.58-2.23	1.60
	Dark	0.17-1.79	1.00

Data extracted from J. W. White (1978)

3.1.4. HYDROXYMETHYLFURFURAL CONTENT OF HONEY

Hydroxymethylfurfuraldehyde or HMF is a decomposition product of fructose. In fresh honey it is present only in trace amounts and its concentration increases with storage and prolonged heating of honey (S. Bogdanov, 2002). Honey is heated at different stages of its processing to

reduce viscosity, destroy yeast, and dissolve crystals. However, such heat treatments increase the HMF content of honey. Therefore, HMF content can be used as an indicator to detect the heat damage and adulteration of honey. Further, it has been reported that the HMF content of honey increases during storage in the warm climates of tropical and subtropical countries. The latest Codex standards for the HMF content of honey is set as less than 40 mg/kg after processing and/or blending of honey. However, a standard for HMF of less than 80 mg/kg has been set for the honeys produced in countries or regions with tropical ambient temperatures and in blends of these honeys (CODEX STAN 12-1981, 2001; EC 2001/110).

3.1.5. ACIDITY OF HONEY AND pH

The acid content of honey is relatively low but it is important for the honey taste. Most acids are added by the bees (T. Echigo and T. Takenaka, 1974). The main acid is gluconic acid, a product of glucose oxidation by glucose oxidase. However, it is present as its internal ester, a lactone, and does not contribute to honey's active acidity. Honey free acidity is determined by titration (S. Bogdanov, 1997) and is expressed in milli equivalents per kg. The comparative values for rosemary honey is 17.2 ±0.4 meq/kg (C. Pérez-Arquillué *et al.*, 1995), 15.9 ±1.5 meq/kg (E. Mendes *et al.,* 1998) and 17.2-20.5 meq/kg (I. Eschrique *et al.,* 2009). The Codex Stan for honey sets the maximum level below 50 meq/kg. The following acids have been found in minor amounts: formic, acetic, citric, lactic, maleic, malic, oxalic, pyroglutamic and succinic (I. Mato *et al.,* 2003). Most honeys are acidic, that means that the pH value is smaller than 7. The pH of blossom honeys varies between 3.3 to 4.6. The pH of rosemary honey is 3.71 with a range of 3.42-3.99 (C. Pérez-Arquillué *et al.*, 1995; I. Eschrique *et al.,* 2009).

3.1.6. MOISTURE CONTENT

The moisture content plays an important role in the stability of honey in relation to fermentation and granulation during storage. Normally, the moisture content of honey is below 18.6 % (B. d'Arcy, 2007; J. J. A. Snowdon and Dean O Cliver, 1996; E. Crane, 1976). J. W. White (1975) quoted the work by A.G Lochhead (1933) who reported that honeys with moisture content below 17.1% will not ferment and above 20 % are liable to ferment. However, honey with moisture content between 17.1-20.0 % will ferment depending on the yeast counts in the honey. The Spanish Official State Bulletin (BOE-A-1986-15960) accepts maximum moisture contents of

17.5 to 18.5 g/100 g honey. The analysis of rosemary honeys by E.Mendes *et al.* (1998) found that the average moisture content of honey is 15.2 ± 1.0 % with a range of 13.6 – 16.1 %. A recent study of Portuguese rosemary honeys showed that the moisture content level was 16.5-18.1 % (I. Escrhique, 2009). C. Pérez-Arquillué *et al.* (1995) reported moisture content of 15.4-18-3 % in their analysis of Spanish rosemary honeys.

Honey's water activity varies between 0.5 (16 % moisture) and 0.6 (18.3 % moisture) in the 40-100 °F (4-37 °C) temperature range.

3.1.7. AROMA COMPOUNDS, TASTE-BUILDING COMPOUNDS AND POLYPHENOLS

Honey flavour is an important quality for its application in food industry and also a selection criterion for consumer's choice. Rosemary honey is produced from *Rosmarinus officinalis* (Lamiaceae). This honey has a very good consumer acceptance and commercial value in European countries because of its mild favour and light colour (D. Arraez-Roman *et al.,* 2006).

There is a wide variety of honeys with different tastes and colours, depending on their botanical origin (S. Bogdanov, 2002). The sugars are the main taste-building compounds. Generally, honey with high fructose content (e.g. acacia) are sweet compared to those with high glucose concentration (e.g. rape). Beyond sugars the honey aroma depends on the quantity and quality of honey acids and amino acids. In the past decades some research on honey aroma compounds has been carried out and more than 500 different volatile compounds have been identified in different types of honey. Indeed, most aroma building compounds vary in the different types of honey depending on its botanical origin (S. Bogdanov, 2002).

Polyphenols are another important group of compounds with respect to appearance and functional properties. Phenolic acids and polyphenols are plant-derived secondary metabolites. However, considerable differences in composition and content of phenolic compounds between different unifloral honeys were found. Dark coloured honeys are reported to contain more phenolic acid derivatives but less flavonoids than light coloured ones.

In a study of Spanish rosemary honey thirty-two different volatile compounds were identified (I. Eschrique *et al.,* 2009). The most abundant compounds were: 2-methyl-2-propanol; acetic acid; 2-methyl-3-buten-2-ol and octane. The first three have been previously reported as being present in rosemary honey (C. Pérez-Arquillué *et al.*, 1995; J. S. Bonvehí and F.V. Coll, 2003).

Polyphenols in honey are mainly flavonoids (e.g. quercetin, luteolin, kaempferol, apigenin, chrysin, galangin), phenolic acids and phenolic acid derivatives. The polyphenols are responsible for the antioxidant properties of honey. Rosemary honey phenol content ranges from 1–25 mg/kg of honey (D. Arraez-Roman *et al.,* 2006). The flavonoid content, expressed as mg quercetin equivalents in 100 g of honey (mg QE/100 g), of rosemary honey can vary between 1.00 and 2.35 mg QE/100 g (F.A. Tomás-Barberán *et al.,* 1993a;b).

3.1.8. HONEY COLOUR

Honey colour is measured on a Pfund Grader by the honey industry. It provides continuous readings over the entire colour range of honey. The Pfund colour grader visually compares a standard amber-coloured glass wedge with liquid honey contained in a wedge-shaped cell. The colour intensity of the honey is expressed as a distance (in mm) along the amber wedge and usually ranges between 1 and 140 mm. Rosemary is classified as very light yellow colour, white, with a max 35 mm Pfund.

Table 3. Pfund Grader colour designations.

Colour range Pfund Scale millimetres	Colour Standard designation
0-8 mm	Water White
Up to 17 mm	Extra White
Up to 34 mm	White
Up to 50 mm	Extra Light Amber
Up to 85 mm	Light Amber
Up to 114 mm	Amber
Over 114 mm	Dark

However the colour of honey can be assessed by a number of other methods. For example, the method adopted by the Association of Official Analytical Chemists uses a Lovibond 2000 visual comparator (AOAC, 1990). Studies have also shown that honey colour can be assessed by the CIE- 1931 or the more recent CIE-1976 (L*a*b) or CIELAB methods (R. M. Castro *et al.*, 1992)

It should be note that honey colour it is not an index of quality. Colour is not included in the current USDA grading methods. However, colour is an important characteristic of upon which honey is classified by honey producers, packers and end-users. An estimated 75% of industrial users of honey include colour designations in their specifications (NHB[d], nd).

The colour of honey is characteristic of its floral source due to minerals and other minor components. Exposure to heat and storage time may affect honey's colour (NHB[d], nd). Honey appears lighter in colour after it has granulated. The colour of a specific sample of honey after it granulates depends on the crystal size. The final crystals give the lightest appearance. For this reason, most creamed honeys are opaque and light in colour. Honey can become darker as a result of storage, although at widely differing rates. This depends upon the composition of the honey (acidity, nitrogen, and fructose contents) and its initial colour. Generally, the darkening of honey is temperature sensitive and occurs more rapidly when honey is stored at high temperatures (NHB[d], nd).

3.2.   Microorganisms in honey

Microorganisms found in honey have been identified by many authors (P.C. Molan, 1992; J. W. White, 1963).As it can be seen in Table 4 the microbes of concern are primarily yeasts, moulds and spore-forming bacteria. However, most bacteria and other microbes cannot grow or reproduce in honey i.e. they are dormant and this is due to antibacterial activity of honey.

Table 4. Microorganisms found in honey.

Bacteria	Yeasts	Moulds
Alcaligens	*Ascosphaera*	*Asperhillus*
Achromobater	*Debaromyces*	*Alihia*
Bacillus	*Hansenula*	*Bettsia alvei*
Bacteridium	*Lipomyces*	*Cephalosporium*
Brevibacterium	*Nematospora*	*Chaetomium*
Citrobacter	*Oosporidium*	*Coniothecium*
Clostridium	*Pichia*	*Hormiscium*
Enterobacter	*Saccharomyces*	*Peronsporoceae*
Escherichia	*coli*	*Peyronelia*
Erwinia	*Trichosporium*	*Schizosaccharomyces*
Flavobacterium	*Torula*	*Triposporium*
Klebsiella	*Torulopsis*	*Uredianceae*
Micrococcus	*Zygasaccharomyces*	*Ustilaginaceae*
Neisseria		
Pseudomonas		
Xanthomonas		

adapted from Peter B. Olaitan *et al.,* 2007.

3.3. Processing of honey

Honey processing includes all the handling of honey during which its physical and chemical properties are changed in order to facilitate handling or to improve certain qualities (B. d'Arcy, 2007). It is worth mentioning that each country processes the honey at different temperatures. In this manner, American, Australian and Turkish honey processors reach temperatures above the 65 °C throughout the processing line (E. Crane, 1983; B. d'Arcy, 2007; I. Turhan *et al.*, 2008).

Raw honey is brought into the packing facilities in large barrels directly from beekeepers. At this point, the raw honey is usually in a solid or crystallized state. Before the honey is further received into the system, representative samples from each lot are drawn by the quality personnel who then conduct screening tests usually in their in-house laboratories.

The first step is to homogenise the honey from the different 200 L barrels/drums in a homogenizer tank. This is the first obstacle encountered by the honey processors. Honey is in a solid state and that makes difficult to pour it from the barrel into the homogenization tank. One

alternative is to introduce a resistance (heater) in the barrel to heat and melt the honey, and decant it then into the tank. However this method damages considerably the honey quality as the resistance reaches high temperatures. Furthermore it is an expensive method in terms of power consumption.

Once in the homogenization tank, a gentle heating process is applied in order to slowly begin to liquefy the honey so that it will flow through the filtration system and eventually onto the packaging line. In medium and large-scale continuous flow systems, honey is clarified by passing through a 3 m baffle tank with the jacket of the tank able to be warmed by a suitable method. Moreover, the tank is equipped with a worm screw along the deposit to ensure the continuous homogenization of honey. While it is necessary to filter the raw honey in order to remove any foreign matter, it is important to apply a "respectful" process that minimally alters the pure honey from its natural state. Honey is heat sensitive; therefore honey processors apply no more than is necessary in order to create a liquid, shelf stable product. However, this is a power and time-consuming process; honey is heated at 40-45 °C for 48 hours prior pumping. Upon completion of the filtration process, the honey flows into the packaging system where it is bottled, labelled, coded and boxed for outbound shipment. These are also energy intensive processes. Picture 2 schematizes the of honey processing line.
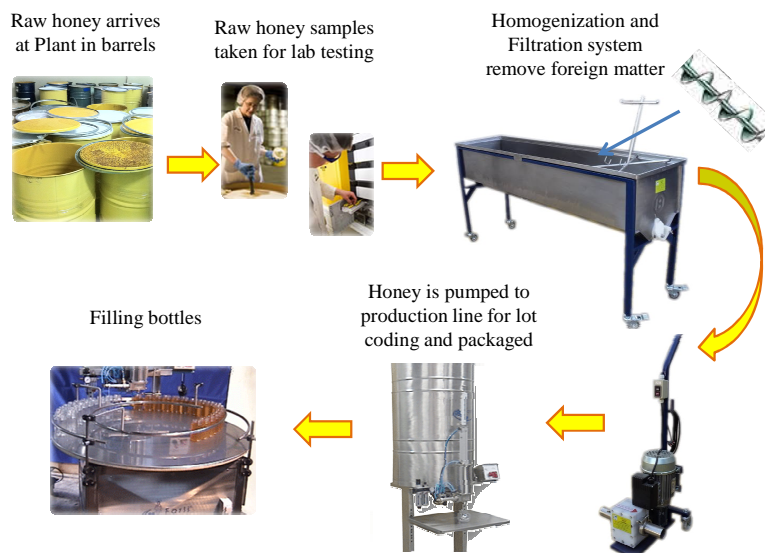
Picture 2. Honey processing line

Storage conditions of honey are also important factors to consider. Honey is subjected to fermentation, granulation (crystallisation), discolouration, flavour damage, and destruction of enzymes and production of hydroxymethylfurfural during storage (B. d'Arcy, 2007). The recommended storage temperature for processed honey is 18-24 °C, and unprocessed honey is below 10 °C (Anonn, 2003; G.E. Marvin, 1930).

The major problem during storage is the fermentation of honey. All honeys with more than 20.0 % moisture are liable to ferment. Honey with less than 17.1 % moisture is not liable to ferment as microorganisms (e.g. yeast) are unable to grow due to the low water activity. However, honey containing moisture content between 17.1 and 20.0 % is fermentable, subject to the amount of yeast present. Honey is safe, if the yeast content is less than 1000 per g for a moisture content of 17.1 %-18.0 % (B. d'Arcy, 2007). Honeys with a moisture content of 18.1 % to 19.0 % and 19.1 % to 20.0 % are not fermentable if the yeast count does not exceed 10 per g and 1 per g respectively. Further, storing honey below 11 °C is reported to prevent fermentation and granulation.

Studies on the storage temperature of honey has shown that a reduction of 5-9 °C in temperature results in a reduction in the rate of HMF production to 1/3 and maximum enzyme loss to 1/5 (J. W. White *et al.,* 1964 cited by B. d'Arcy). Honeys first stored at 0 °C for at least 5 weeks and then at 14 °C, have been shown not to crystallise for 2 years. However, honeys stored only at 14 °C have crystallised within 5 weeks (G.F. Townsend, 1976). Studies on heating honey at 60 °C for half an hour, followed by storage at room temperature (7-30 °C), 5 °C and 40 °C for periods of 0, 2, 4 and 6 months, have shown that honey stored at 40 °C resulted in deterioration of colour but no granulation. Further, there was no difference in the colour between honey stored at room temperature and 5 °C (J. K. Gupta *et al.,* 1992).

3.4.   Honey processing techniques

As mentioned before, in industry the majority of liquefaction and prolongation of the crystallization of honey is carried out by heating methods. The recommended pasteurisation method includes flash heating to 170 °C for a few seconds, 77 °C for few minutes, or heating at 63 °C for 30 min (Anonn, 2003; G. F. Townsend, 1976; J. W. White *et al.,* 1963). Pasteurization delays crystallization by dissolving crystals in the honey by heat treatment and it also kills yeast

cells, which in turn prevents the possibility of fermentation. The main limitation of heating is however, the possibility of quality deterioration.

Due to the limitations of heat treatment, alternate technologies to innovate in the beekeeping sector in terms of processing techniques have been studied in the past. Research effort has focussed on non-thermal alternatives, such as microwave radiation, infrared heating, ultrafiltration and ultrasonics. However, all studies and tests conducted so far have only been performed at laboratory scale. One of the reasons is the lack of continuity of the investigations in order to rescale from laboratory to industrial dimension. On the other hand, the implementation of these new technologies represents a new and important investment in machinery, for which the honey packers are not very likely to make. Hence, the importance of developing a cost-effective method for the treatment of crystallised honey, which would enable processors to liquefy honey to facilitate its decanting and flow in processing and packing plants, as well as to extend its shelf-life and safeguard its quality. The most important technologies are described below.

3.4.1. IRRADIATION

Food is submitted to gamma irradiation process for different purposes. Among the several benefits, a food or an ingredient is irradiated to reduce microorganism's load.

W. Migdal *et al.,* (2000) studied the degree of microbiological decontamination, organoleptic and physico-chemical properties of natural honeys after radiation treatment. Honeys were irradiated with the beams of 10 MeV electrons from a 10 kW linear accelerator at the dose 10 kGy[1]. The time and temperature conditions thought are not mentioned in the paper. It was found that irradiation decreased the amount of aerobic and anaerobic bacteria and fungi by an average of 99 %. Colour, taste, acidity, diastase activity and HMF content did not change radically either. This technology however, is not affordable as the costs of processing are quite high and it is required special permission to produce ionizing radiation at industrial premises.

R. S. Barhate *et al.,* (2003) explored the physico-chemical characteristics and microbiological quality of honey after applying infrared radiation. A near infrared (NIR) batch oven fitted with

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[1] Kilogray is a derived metric (SI) measurement unit of absorbed radiation dose of ionizing radiation (e.g. X-rays). The kilogray is equal to one thousand gray (1000 Gy), and the gray is defined as the absorption of one joule of ionizing radiation by one kilogram (1 J/kg) of matter, e.g. human tissue.

infrared lamps (1.0 kW, peak wavelength 1.1– 1.2 µm) fabricated at the authors institute was used. The samples were heated for a known period (2, 3, 4, 5 and 8 min) and were mixed intermittently (once every 30 s) for 2 s inside the oven for uniform heating. But all treatments increased the HMF and decreased the diastase activity. The peak temperature rose to 100 °C in just 8 min decontaminating the sample but inactivating completely the diastase activity and increasing significantly the HMF content. Even milder conditions changed the quality parameters of honey.

F.S. Sabato (2004) studied both rheological and sensorial properties of two varieties of Brazilian honey when they were irradiated at 5 and 10 kGy. The viscosity was measured at four temperatures (25 °C, 30 °C, 35 °C and 40 °C) for both samples, and compared with control and within the doses. The results they obtained were that the gamma irradiation did not impair the viscosity in the studied doses (5 and 10 kGy) and they did not differ significantly from the control ($p < 0.05$) for both types of honey. Regarding the sensorial evaluation, there was a significant difference for some parameters due to the irradiation doses, being the taste significantly affected ($p < 0.05$). So, although the gamma irradiation is an effective treatment for the decontamination of bacteria and fungi, it does not help to the handling, packing or processing of honey and it also affects to the sensorial properties.

A. Bera *et al.,* (2009) subjected honey to gamma radiation with source of cobalto-60 (10 kGy) at environmental conditions and studied the physico–chemical characteristics of irradiated honeys according Brazilian regulations. The authors however, do not describe the treatment conditions (time and temperature). Their results showed that gamma radiation, in the dose mentioned above, did not cause significant physicochemical alterations.

3.4.2. MICROWAVE AND INFRARED HEAT

Infrared (IR) heating can be applied to various food processing operations, namely, drying, baking, roasting, blanching, pasteurization, and sterilization. Combinations of IR heating with microwave heating and other common conductive and convective modes of heating have been gaining momentum because of increased energy throughput (K. Krishnamurth *et al.,* 2008).

R. S. Barhate *et al.,* (2003) processed the honey by applying microwave and explored their effect on the physico-chemical characteristics and microbiological quality. Microwave heating studies were conducted in a micro-convective oven with turntable attachment. Experiments were carried

out at different power levels (PL) ranging from 10 to 100 (175–850 W) and for different heating periods of 15 to 90 s. The corresponding microwave input power intensities are expressed in terms of W/g (3.5, 6.3, 9.1, 11.9 and 16.0 W/g corresponding to 10, 30, 50, 70 and 100 PL respectively. Their results indicated that the extent of change in honey properties mainly depended on the power level (power intensity) and duration of heating. Changes were prominent in samples that were heated at higher power levels and for longer durations. Moreover, the peak temperature attained by the sample depended on the power level used as well as duration of heating. Samples presented a lower yeast contamination than the control sample. The product temperature measured at the end of the heating period (peak temperature) though, increased so that it rose to 110 °C, decreasing the diastase activity and increasing the HMF concentration. Hence, even that microwaves are effective for the decontamination of yeasts, it also affects the honey quality parameters.

Z.W. Cui *et al.,* (2007) investigated the effect of microwave-vacuum (MWV) drying as a potential method for obtaining high-quality dried honey. Liquid honey was heated and dehydrated in a MWV dryer to a moisture content less than 2.5 % within about 10 min. The drying curves and the temperature changes of samples were tested during MWV drying at a different of microwave power, vacuum pressure levels and sample thicknesses. A sample thickness of less than 8 mm and a vacuum pressure of 30 mbar were identified as the better parameters for the MWV drying. The core temperatures of the sample were about the same as the surface temperatures, the temperature changes were from 30 to 50 °C with higher dehydration rates while no darkening of the honey took place during MWV drying. There were no significant changes on the contents of fructose, glucose, maltose and sucrose in the honey after MWV drying. The volatile acids, alcohols, aldehydes and esters made up the bulk of the identified aroma compounds of the used liquid honey and the content of alcohols and the esters changed slightly. The acids decreased markedly whereas the aldehydes and the ketones increased remarkably in the honey dehydrated by MWV drying. However, this method is very expensive and cannot be used as a common method for processing honey.

3.4.3. MICROFILTRATION

Filtering removes bee parts, wax and solids, including the majority of pollen that can hasten crystallization.

Much commercial honey is micro-filtered, often using natural diatomaceous earth (DE) process to eliminate even micron-sized particles. DE is the remains of microscopic one-celled plants (phytoplankton) called diatoms that lived in the oceans. Diatomaceous earth is mined and has several important uses as a filtering material for foods and beverages. DE is approximately 3% magnesium, 86% silicon, 5% sodium, 2 % iron and has many other trace minerals such as titanium, boron, manganese, copper and zirconium. The pore size ranges from 0.5– 22 micrometers (µm).

DE is often used to filter honey as it functions to reduce non-honey (i.e. wax, pollen, bee parts, wood chips, and some bacteria) particulate matter. Most pollen, wax and some bacteria can be removed through the use of DE. This helps remove the presence of any particulate and produces a very clear end-product. In order to use DE, honey needs to be heated slightly to allow it to pass through the micropores. The use of DE for filtering honey is regulated in the EU depending upon the level of filtration. According to the EU, if DE is used to thoroughly remove the pollen from honey, it may be difficult to identify the botanical and geoFigureal origin. This level of filtration also makes it difficult to identify other microscopic elements normally found in honey (NHB, 2004).

R. S. Barhate *et al.,* (2003) also studied the effect of using membrane technology to produce a honey that is free of microorganisms and suspended matter. Their ultrafiltration (UF) results indicate that the UF membranes completely reject enzymes and totally eliminate yeast cells in honey. There was no diastase activity found in the permeates of UF membranes (20, 25, 50 and 100 KDa). The rejection of lower molecular weight enzymes gives a clear indication that enzymes of higher molecular weights are also rejected by these membranes, so as the ultrafiltered honey is devoid of desirable enzymes and proteins it cannot be regarded for applications related to health foods. The authors propose applications of ultrafiltered honey in gel formulations, cosmetics and pharmaceutical preparations and as sweetener in tea/coffee and fruit beverages, but not for the honey itself.

3.4.4. ULTRASONICATION

Ultrasonication is a processing alternative for many liquid food products. Its mechanical power is being used for a gentle yet effective microbial inactivation and particle size reduction. When honey is exposed to ultrasonication, most of the yeast cells are destroyed. Yeast cells that survive

sonication generally lose their ability to grow. This reduces the rate of honey fermentation substantially. Ultrasonication does also eliminate existing crystals and inhibit further crystallization in honey.

T. Kalogereas (1955), Liebl (1978) and B. D'Arcy (2007) reported that high- frequency sound waves (9, 18, 23 and 24 kHz) eliminated the existing crystals and retarded further crystallization. They also revealed that high frequency sound destroys the yeast, improves the appearance and inhibits the granulation of the product.

T. Kalogereas (1955) investigated the use of high frequency ultrasound waves on honey. However, the procedure was abandoned as expensive, since the treatment periods demanded a relatively longer time period of 15-30 min at 9 kHz. Yet, ultrasonic waves at 18 kHz, were found to liquefy 1500 kg of honey in 1 h. Further studies at higher frequencies (23 kHz) were conducted and found that ultrasonic treatment resulted in a lower HMF content compared to heating and none of the chemical parameters tested were significantly affected. The samples remained in a liquid state for a longer period from the ultrasonic waves than the heat procedure.

S. Kai (2000) studied the ultrasonic liquefaction at a frequency of 20 kHz of Australian honeys (Brush box, Stringy bark, Yapunyah and Yellow box) and compared the results with heating in a water bath and an oven. It was observed that the sonication at a frequency of 20 kHz of 150 g of stringy bark honey for 3 min completely disintegrated the honey crystals (i.e. liquefied the honey). Neither the water bath nor the oven set at 70 °C were capable of liquefying 150 g of honey in 8 and 34 min, respectively, as efficiently as the ultrasonic method. Ultrasonically treated samples remained in liquefied state for approx. 350 days, an increase of 20 % when compared to heat treatment. However, no comparison between the various treatments was concluded in terms of the HMF and it was concluded that the method of analysis may not have been sufficiently sensitive to detect the quantity of HMF. It was also observed that different honey types required different levels and times of sonication to attain the same level of crystal disintegration.

Already in 1976, Liebl patented a "Method on preserving honey", which claims a method for treating honey by exposing the honey to ultrasound of at least 18 kHz in a temperature range of 10 to 38 °C for less than 5 minutes whereby crystallisation and fermentation of the honey are inhibited. No additional patents dealing with the application of ultrasound in honey processing exist.

Hielscher, a German SME developing and producing ultrasound equipment for various applications, has a work on the application of ultrasound to honey processing that summarises the available literature on the topic. However, no specific equipment is offered and the article ends with the recommendation to conduct trials using a bench-top size sonication system in batch mode, while further processing trials require a flow cell for pressurised recirculation or in-line testing (Hielscher, n.d).

3.5. Ultrasounds in food processing

Ultrasound (US) is acoustic (sound) energy in the form of waves having a frequency above the human hearing range. The highest frequency that the human ear can detect is approximately 20.000 Hz. This is where the sonic range ends, and where the ultrasonic range begins.

These waves are transmitted through a medium (water) without being weakened in power. They cause mechanical and thermal changes in the material through which they pass, and cause changes in unicellular organisms in addition to other effects. In fact, due to their attributes, they have recently (<5 years) become an efficient tool for large-scale commercial applications, such as emulsification, homogenization, extraction, crystallization, dewatering, low temperature pasteurization, degassing, defoaming, activation and inactivation of enzymes, particle-size reduction and viscosity alteration (A. Patist and D. Bates, 2008). The main applications of the US technology are summarized in Table 5.

Table 5. High-power ultrasound applications in the food industry.

Application	Mechanism	Benefit
Extraction	Increased mass transfer of solvent, release of plant cell material (cavitation dislodgement)	Increased extraction efficiency, yield in solvent, aqueous or supercritical systems
Emulsification/ Homogenization	High shear micro-streaming	Cost effective emulsion formation
Crystallization/ De-crystallization	Nucleation and modification of crystal formation	Formation of smaller crystals. Crystals breakdown
Filtration/screening	Disturbance of the boundary layer	Increased flux rates, reduced fouling
Separation	Agglomeration of components at pressure nodal points	Adjunct for use in non-chemical separation procedures
Viscosity alteration	Reversible and non-reversible structural modification via vibrational and high-shear. Sono-chemical modification involving cross-linking and restructuring	Non-chemical modification for improved processing traits, reduced additives, differentiated functionality
Defoaming	Airborne pressure waves causing bubble collapse	Increased production throughput, reduction or elimination of antifoam chemicals, and reduced wastage in bottling lines.
Extrusion	Mechanical vibration, reduced friction	Increased throughput
Enzyme and microbial inactivation	Increased heat transfer and high shear. Direct cavitational damage to microbial cell membranes	Enzyme inactivation adjunct for improved quality attributes
Fermentation	Improved substrate transfer and stimulation of living tissue, enzyme processes	Increasing production of metabolites, and acceleration of fermentation processes
Heat Transfer	Improved heat transfer through acoustic streaming and cavitation	Acceleration of heating, cooling and drying of products at low temperature

3.6. Mechanism of ultrasound action

The fundamental effect of ultrasound on a continuum fluid is to impose an acoustic pressure (P) in addition to the hydrostatic pressure already acting on the medium. The acoustic pressure often is a sinusoidal wave dependent on time (t), frequency (f) and the maximum pressure amplitude of the wave (P_{max}):

$$P(t) = P_{max} \sin(2\pi ft)$$

The maximum pressure amplitude of the wave (P_{max}) is directly proportional to the power input of the transducer.

At low intensity (amplitude), the pressure wave induces motion and mixing within the fluid, so called acoustic streaming (T. G. Leighton, 2007). At higher intensities, the local pressure in the expansion phase of the cycle falls below the vapour pressure of the liquid, causing tiny bubbles to grow (created from existing gas nuclei within the fluid). A further increase generates negative transient pressures within the fluid, enhancing bubble growth and producing new cavities by the tensioning effect on the fluid (T. J. Mason *et al.*, 1992). During the compression cycle, the bubble shrinks and their contents are absorbed back into the liquid. However, since the surface area of the bubble is now larger, not the entire vapour is absorbed back into the liquid and thus the bubble grows over a number of cycles (Picture 3).

Within a critical size range the oscillation of the bubble wall matches that of the applied frequency of the sound waves causing the bubble to implode during a single compression cycle (A. Patist and D. Bates, 2008). Process of compression and rarefaction of the medium particles and the consequent collapse of the bubbles comprises the well-known phenomenon of cavitation, the most important effect in high power ultrasonics. The conditions within these imploding bubbles can be dramatic, with temperatures of 5000 K and pressures of up to 1000 atmospheres, which in turn produces very high shear energy waves and turbulence in the cavitation zone (A. Patist and D. Bates, 2008). It is the combination of these factors (heat, pressure and turbulence) which is used to accelerate mass transfer in chemical reactions, create new reaction pathways, break down and dislodge particles (when cavitation in proximity of a solid surface) or even generate different products from those obtained under conventional conditions.
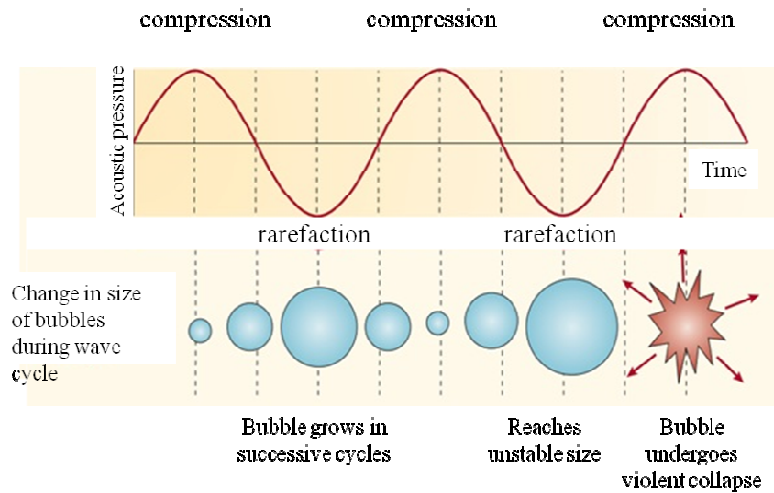
Picture 3. Cavitation phenomenon

Ultrasonic cavitation is a physical phenomenon whose performance depends upon the parameters described below:

Frequency

At high sonic frequencies, on the order of the MHz, the production of cavitation bubbles becomes more difficult than at low sonic frequencies, of the order of the kHz. To achieve cavitation, as the sonic frequency increases, so the intensity of the applied sound must be increased, to ensure that the cohesive forces of the liquid media are overcome and voids are created. The physical explanation for this lies in the fact that, at very high frequencies (Picture 4), the cycle of compression and decompression caused by the ultrasonic waves
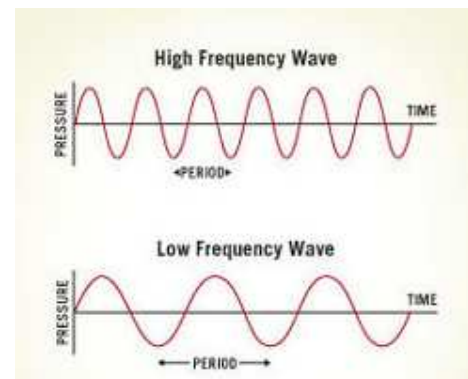
Picture 4. Ultrasound frequencies

becomes so short that the molecules of the liquid cannot be separated to form a void and, thus, cavitation is no longer obtained (J.L. Capelo-Martinez, 2008).

Intensity

The intensity of sonication is proportional to the amplitude of vibration of the ultrasonic source and, as such, an increment in the amplitude of vibration will lead to an increase in the intensity of

vibration and to an increase in the sonochemical effects. To achieve the cavitation threshold a minimum intensity is required. This means that higher amplitudes are not always necessary to obtain the desired results. In addition, high amplitudes of sonication can lead to rapid deterioration of the ultrasonic transducer, resulting in liquid agitation instead of cavitation and in poor transmission of the ultrasound through the liquid media.

Solvent

The solvent used to perform sample treatment with ultrasonication must be carefully chosen. As a general rule, most applications are performed in water. However, other less polar liquids, such as some organics, can be also used, depending on the intended purpose. Both solvent viscosity and surface tension are expected to inhibit cavitation. The higher the natural cohesive forces acting within a liquid (e.g., high viscosity and high surface tension) the more difficult it is to attain cavitation.

Temperature

Solvent temperature plays two roles in ultrasonication. On the one hand, the use of high temperatures helps to disrupt strong solute–matrix interactions, which involve Van der Waals forces, hydrogen bonding and dipole attractions between the solute molecules and active sites on the matrix. Moreover, faster diffusion rates occur at higher temperatures. On the other hand, cavitation is better attained at lower temperatures when the ultrasonic power of the generator is constant. This is because as the temperature of the solvent rises so too does its vapour pressure and so more solvent vapour fills the cavitation bubbles, which then tend to collapse less violently, that is, the sonication effects are less intense than expected. Hence a compromise between temperature and cavitation must be achieved.

3.7. Equipment for the generation of high-power ultrasound

Ultrasound can be divided into three frequency ranges; power ultrasound (16–100 kHz), high-frequency ultrasound (100 kHz–1 MHz) and diagnostic ultrasound (1–10 MHz). The work published by (T.J. Mason *et al.*, 1996) shows that the frequency is inversely proportional to the bubble size. Therefore, low frequency ultrasound (that is, power ultrasound 16–100 kHz) generates large cavitation bubbles (vapour bubbles) resulting in higher temperatures and pressures in the cavitation zone. As the frequency increases, the cavitation zone becomes less

violent and in the megahertz range no cavitation is observed at typical amplitudes and the main mechanism is acoustic streaming. While medical imaging operates at frequencies in the megahertz range, most industrial applications (processing of chemicals, food as well as cleaning) operate between 16 and 100 kHz because cavitation can be produced more easily at this frequency range.
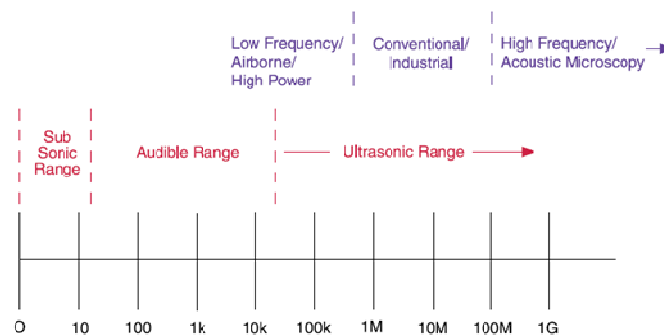
Picture 5. Acoustic spectrum breaks down sound intro 3 ranges of frequencies. The US range is then broken down further into 3 sub sections.

The use of ultrasonics in industrial processes has two main requirements: a liquid medium and a source of high-energy vibrations. The vibrational energy source is called a transducer, which transfers the vibration (after amplification) to the so-called sonotrode or probe, which is in direct contact with the processing medium.

At present there are three classes of ultrasonic baths (H. M. Santos *et al.*, 2007). The classic one is the common ultrasonic bath, which is found in most laboratories. This bath works with only one frequency, generally 40 kHz, and can be supplied with temperature control. A second type is provided as a multifrequency unit, which operates using, simultaneously, ultrasonic transducers with different frequencies, for instance 25 and 40 kHz, on the bottom and the side, respectively. The benefit of this is a uniform ultrasonic power distribution (Bandelin electronics, 2012). The third model corresponds to the most advanced in terms of technology, including the following features (Elma ultrasonics, 2011):

1. Dual frequency of sonication.

2. A choice of 25/45 or 35/130 kHz. The baths are designed to work with one of the two frequencies at a time.

3. Power regulation.

4. The intensity of sonication can be controlled through amplitude control (10–100%).

5. Three operation modes:

   (a) Sweep: in this mode the frequency varies within a defined range. In this manner the ultrasonic efficiency is more homogeneously distributed in the bath than during standard operation.

   (b) Standard

   (c) Degas: the power is interrupted for a short period so that the bubbles are not retained by the ultrasonic forces.

6. Heating and timer.

4. CHAPTER 1: ULTRASOUNDS ON HONEY LIQUEFACTION
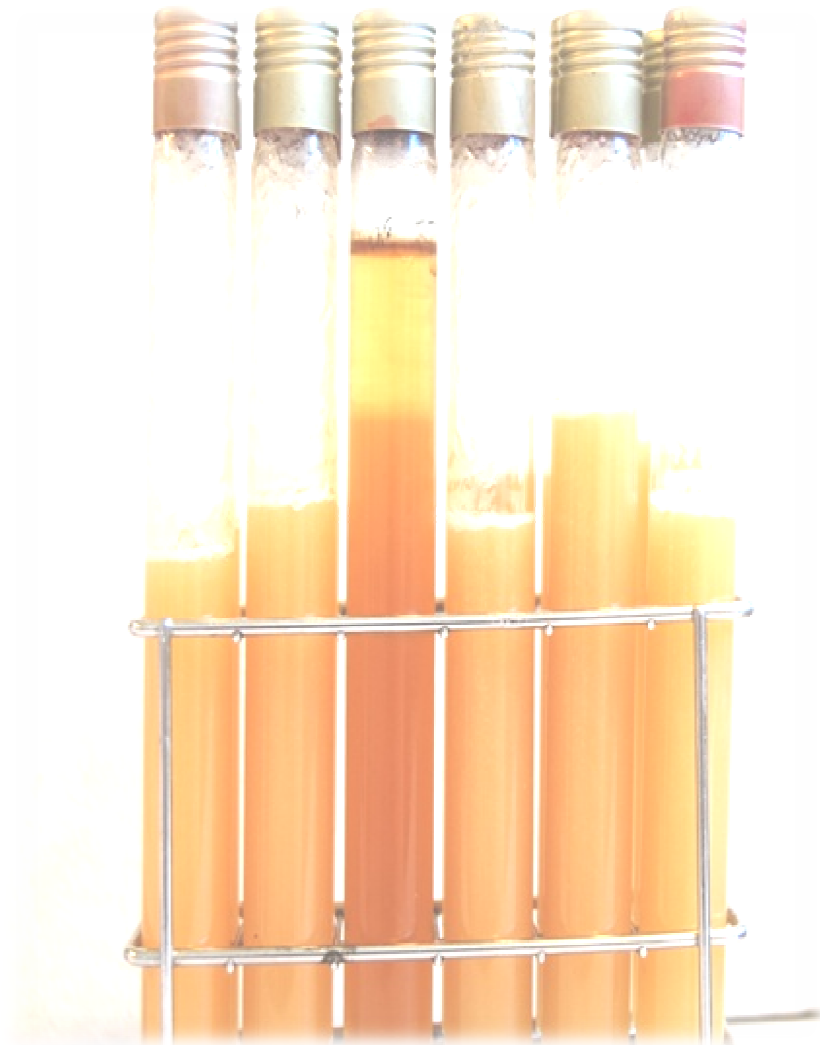
4.1.   Introduction

Honey is a supersaturated solution of glucose and it has a **tendency to crystallize** spontaneously at room temperature in the form of glucose monohydrate. Crystallization of honey, also called granulation, is an undesirable process in liquid honey because it affects the textural properties, making it less appealing to the consumer, who prefers it liquid and/or transparent (E. Tosi *et al.*, 2004; E. Saxena *et al.*, 2009; S. Ajlouni and P. Sujirapinyokul, 2009). Moreover, the **crystallization of honey usually results in increased moisture of the liquid phase, which makes the honey vulnerable to yeast growth that may cause fermentation** of the product and, within time, produces subsequent **organoleptic modifications** and **degrades the quality**. Crystallization also **causes problems during handling and processing**. Machines cannot work properly due to the high viscosity, ''stickiness'' and complexity of the fluid. Different types of honey will crystallize at different rates. Some honey crystallizes within a few weeks after extraction from the combs, whereas others remain liquid for months or years. The main factors that influence

the speed of crystallization according to Khalil Hamdan (n.d) are:

(i) the nectar source collected by bees (the sugar composition of honey),

(ii) the methods in which honey is handled (processed) and

(iii) the temperature in preservation.

The time it will take the honey to crystallize depends mostly on the ratio of fructose to glucose, the glucose to water ratio. Honey high in glucose sugar, with a low fructose to glucose ratio will crystallize more rapidly. Honey with a higher fructose to glucose ratio (containing less than 30% glucose) crystallizes quite slowly and can stay liquid for several years without special treatment. Moreover, the higher the glucose and the lower the water content of honey, the faster the crystallization. Oppositely, honey with less glucose relative to water is a less saturated glucose solution and is slow to crystallize. Table 6 shows the relative speed of crystallization in descending order of various honeys.

Table 6. Relative Crystallization Speed of Various Honeys.

Honey Type	Crystallization	Honey Type	Crystallization
Australian acacia	Very slow	Rosemary	Slow
Black locust (*Robinia pseudoacacia*)	Very slow	Sourwood (*Oxydendrum arboreum*)	Slow
Cranberry	Very slow	Spanish Lavender (*Lavendua Stoechas*)	Slow
Litchi	Very slow	Thyme (*Thymus vulgaris*)	Slow
Longan	Very slow	Alfalfa	Rapid
Milk vetch (*Astragalus*)	Very slow	Apple, pear, plum and cherry	Rapid
Milkweed (*Ascelpia syriaca*)	Very slow	Clover (*Trifolium*)	Rapid
Sage (*Salvia officinalis*)	Very slow	Cotton	Rapid
Sidr/jujube	Very slow	Dandelion	Rapid
Tulip poplar	Very slow	Lavender	Rapid
Tupelo	Very slow	Phacelia	Rapid
Bell heather (*Calluna cinerea*)	Slow	Field bean (*Vicia faba*)	Rapid
Blackberry	Slow	Goldenrod (*Solidago*)	Rapid
Borage (*Borago officinails*)	Slow	Holly (*Ilex aquifolium*)	Rapid
Buckwheat	Slow	Ivy (*Hedera Helix*)	Rapid
Chestnut (*Castania sativa*)	Slow	Mesquite (*Prosopis spp.*)	Rapid
Citrus (Orange blossom honey)	Slow	Mustard	Rapid
Eucalyptus	Slow	Oilseed rape	Rapid
Fireweed (*Epilobium angustifolium*)	Slow	Raspberry	Rapid
Linden/lime/basswood	Slow	Star thistle (*Centaurea solstitialis*)	Rapid
Maple (*Acre spp.*)	Slow	Sunflower	Rapid
Hawthorn (*Crataegus spp.*)	Slow	Wild thyme (*Thymus serpyllum*)	Rapid
Nodding thistle (*Carduus nutans*)	Slow		

Data extracted from Khalil Hamdan (n.d)

When heated (i.e. 62.7 °C for 30 min), honey becomes liquid, but **a thermal treatment also degrades the honey quality** (E. Tosi *et al.*,2002, 2004, 2008; I. Turhan *et al.*, 2008; E. Crane, 1984). Moreover, heat-treated honey still tends to re-crystallize over time. Therefore, is not only important to ensure the liquefaction but to avoid or hinder the re-crystallization of honey too.

Knowledge of the rheological characteristics of honey is very important from storage and handling point of view (H.I Assil *et al.*, 1991). Food rheology is the study of the rheological properties of food, that is, the consistency and flow of food under tightly specified conditions. The consistency, degree of fluidity, and other mechanical properties are important in understanding how long food can be stored and how stable it will remain. It is also important in determining food texture and in quality control during food manufacture and processing. Moreover, the acceptability of food products to the consumer is often determined by food texture, such as how spreadable and creamy a food product is. Thus**, the rheological behaviour of honey is investigated for shelf-life, handling, packing and processing issues**.

Alternate technologies such as ultrasounds (US) have been studied in the past: T. Kalogereas (1955), Liebl (1978), A. Thrasyvoulou *et al.* (1994) and B. d'Arcy (2007) reported that low-frequency sound waves (9,18, 23 and 24 kHz, respectively) eliminate the existing crystals and retard further crystallization. They also revealed that high-frequency sound destroys the yeast, improves the appearance, and inhibits the granulation of the product. However, the use of ultrasonic waves has not been promoted as it might have been. So far, there are no studies on the effects of the changes in its physical and chemical characteristics for rosemary honey type.

In this Chapter, crystallized rosemary honey samples were treated through an ultrasound bath, filled with distilled water, at a nominal frequency of 40 kHz in a temperature range of 40–60 °C. An US bath was used because ultrasonic waves are spread evenly and this prevents the high intense sonication of honey near the transducer. This ensures the homogeneous and less aggressive incidence of the ultrasound waves to honey. Furthermore, the avoidance of high temperatures saves energy consumption.

The present Chapter aimed at evaluating the effect of different combinations of ultrasound treatment (power, temperature and duration) on honey liquefaction; therefore, the rheological properties of honey were evaluated in order to:

- Study the **viscosity** behaviour of ultrasonicated honey. To that end, it will be compared with samples treated by conventional heating.
- Evaluate the honey **crystal content** after an ultrasound treatment.

- Asses the **storage behaviour** of US-treated honey, that is, control the tendency re-crystallization.
- Study the **thermal properties** (glass transition temperature and change of enthalpy) of US honey.

Moreover, to ensure the homogeneity of the ultrasonic bath and to locate the most efficient place inside the bath a foil aluminium test was performed.

4.2. Material and methods

4.2.1. Sample treatment

Rosemary *(Rosmarinus officinalis)* commercially honey was used (Bona Mel Organic, S.L. Organic Production, CE control System, Alicante) for the preliminary tests. The rest of the experiments were performed using rosemary honey obtained from a local producer (Viadiu Ltd., Caldes de Montbuí, Spain).

Samples were placed in 35 ml thin-walled glass test tubes since glass is impermeable, inert, and easily sterilized by heat according to good laboratory practice. The test tubes were then covered with a screw top, wrapped with parafilm and subjected to the following treatments (Picture 6):

a) Liquefaction using an ultrasound (US) bath (Powersonic 603, Cobos Precisions, Korea) operating at a nominal frequency of 40 kHz (input power 200 W) during a maximum period of 120 minutes in a temperature range of 40-50 °C.

b) Liquefaction by only heating (HT) using a thermostatic bath (Digiterm 200 P Selecta S.A, Barcelona, Spain) during a maximum period of 120 minutes in a temperature range of 40-50 °C.
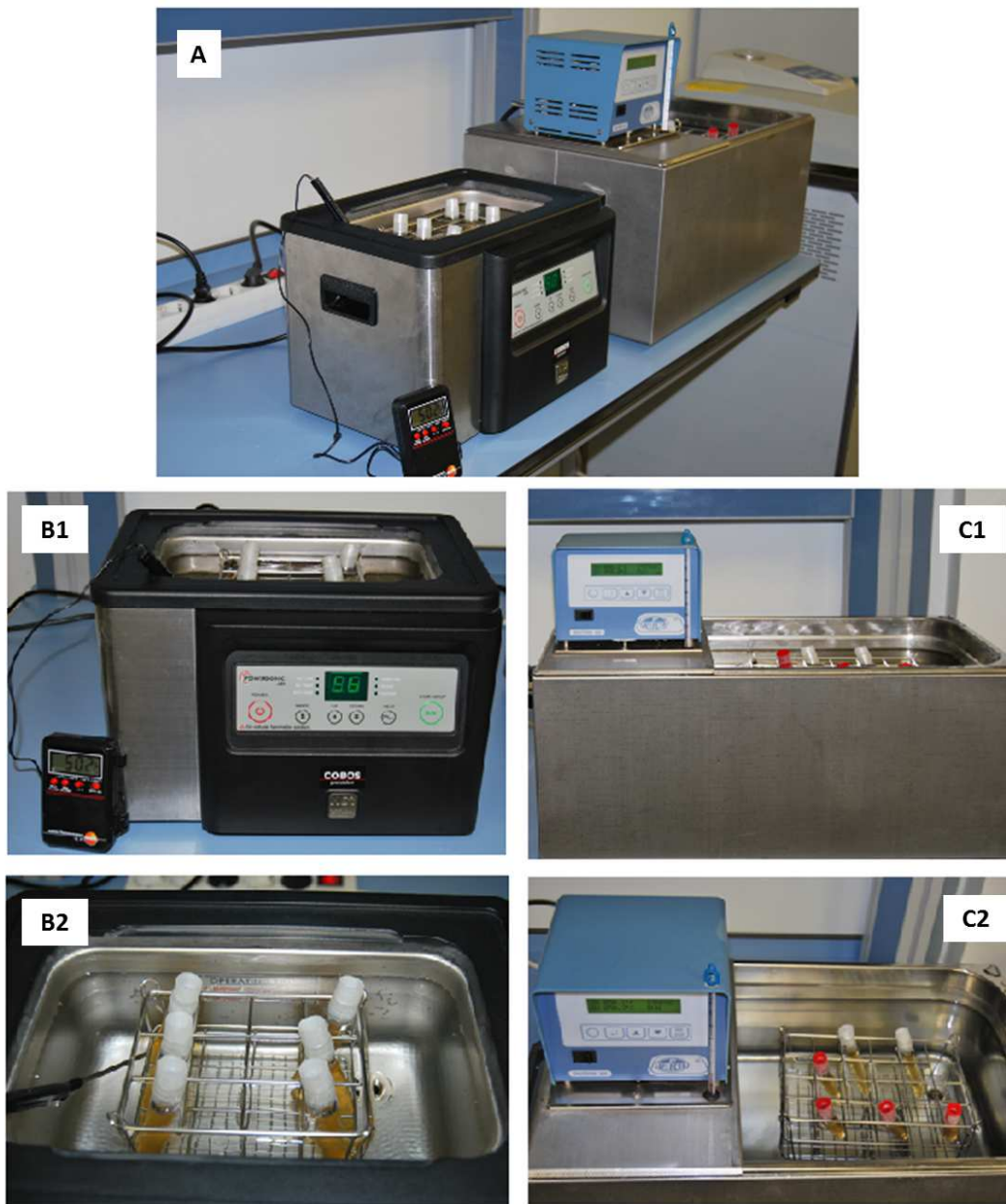
Picture 6. Image of the laboratory equipment for the liquefaction treatments. (A) Ultrasound and Thermostatic baths. (B1) Ultrasonic bath. (B2) Samples in the ultrasonic bath. (C1) Thermostatic bath. (C2) Samples in the thermostatic bath.

4.2.2. Viscosity

Viscosity is the measure of the internal friction of a fluid. The viscosity measurements were carried out using a Brookfield disc-type viscometer (model RVTI, Brookfield Engineering Labs. Inc, Sotughton MA 02072, USA). This type of viscometer consists of a spindle having a flat circular disc near the bottom of the rod. The shape of the disc spindle makes the calculation of shear rate an approximation or average across the surface of the disc. Viscosity in mPa s is then calculated using a conversion table according to the disc-type and the spindle speed applied to the fluid.

The measurements were performed at a 40 °C, 50 °C and 60 °C, and at each temperature after 20, 40 and 60 minutes, both with and without US treatment. The spindle type was RV-2 and the spindle speeds (shear rate in rpm) were 2.5, 5, 10 and 20 rpm. Samples were stored in 250 ml glass jars and all viscosity measurements were undertaken in the sample container to avoid air bubble incorporation during sample transfer and handling. The analyzes were made by triplicate for each sample and the mean viscosity (mPa s) was calculated. Moreover, the viscosity was measured continuously during the first 20 minutes to measure the liquefaction speed in order to quantify the time to achieve a certain value.

Because of the regular dependence of the viscosity of honey on temperature, a linear relationship between viscosity and the reciprocal of absolute temperature can be obtained. Thus, it is possible to apply the Arrhenius equation;

$$\mu\ (T) = \mu_0\ \exp(-E_a/RT) \qquad \qquad \textit{Equation 1}$$

Where μ is the viscosity (mPa s), μ_0 is constant, E_a the activation energy (J mol $^{-1}$), R the gas constant (8.314 J Kg $^{-1}$ K^{-1}) and T the absolute temperature (Kelvin).

A linear plot of ln k_T versus (1/T) will be drawn to obtain the activation energy.

4.2.3. Crystal content observation

The presence/lack of crystals in the treated honey was performed using a microscope as a part of an image analyser (Leica Microsystems DM 2500, Wetzlar, Germany) and a fiber optical light source (Euromex fiber optical light source EK-1 model: LE 5210-230 REV.S, Holland) and the images was recorded using a camera (Leica Microsystems DFC 280, Heerbrugg). A 1 ml aliquot of honey sample was placed in a slide glass fixed with a sample holder.

4.2.4. Storage behavior

As previously described by M.S. Finola *et al.* (2007), honey samples were loaded into a 1 cm path-length cuvette, and the absorbance at 660 nm was measured once a week using a spectrophotometer (Nicolet Evolution e 300, Thermo Electron corporation, England).
On the other, beakers with US and HT-treated honey were stored at 4 °C for two years for visual evaluation.

4.2.5. Differential scanning calorimetry (DSC)

A STAR$^®$ System DSC1 (Mettler-Toledo AG, Analytical, Switzerland) was used for thermal scanning of honey samples. The DSC was attached with a refrigerated cooling system which efficiently controlled and monitored temperature up to -100 °C. The instrument was calibrated with indium at the same scanning rate as the samples. Nitrogen was used to purge gas at a flow rate of 50 mL/min. Samples (5-8 mg) were weighed accurately into aluminium pans, hermitically sealed. A sealed empty aluminium pan was used as reference. The thermal scans were conducted from -100 °C to 250 °C at the scanning rate of 10 °C/min to obtain a complete thermal behaviour of honeys from low temperature to high temperature. The analysis of the plot by STAR$^®$ thermal analysis system version 10.00 software determined the glass transition and the enthalpy change. The glass transition temperature was defined as the point of inflection of the heat capacity change. Enthalpy change (ΔH) for the honey sample was calculated by dividing the area under the peak by the mass of the sample. The enthalpy change is equal to the energy required to dissolve D-glucose monohydrate crystals in honey. Reported data are the average of three determinations.

4.2.6. Location of the highest intensity inside the ultrasonic bath

It is thought that baths spread evenly the ultrasound waves so the homogeneity of the bath is such that samples can be placed anywhere and the effect is perceived equally. However, to determine the transducer's position several sheets of aluminium were placed horizontally (covering entirely the bottom) and vertically in the bath to place the honey samples on that part (so the effect is more perceptible) as Picture 7 shows.

Picture 7. Placing of the aluminum sheets. Right: bottom placing. Left: Vertical placing.

4.2.7. Statistical analysis

Minitab 15 was used to perform statistical analyses of the data obtained. ANOVA was study the effect of ultrasound and heating at different time and temperatures on viscosity and T-test ($\alpha=0.05$) was used to examine for any significant differences between the means of viscosity among these treatments.

4.3.   Results and discussion

4.3.1. Viscosity

For all shear rates investigated (2.5, 5, 10 and 20 rpm), all honey samples showed a Newtonian behaviour; Figure 1 shows the typical relationship between viscosity and shear rate for the rosemary honey as a representative of the trend obtained for ultrasound (US) treated sample (Figure 1 (A)) and heat treated (HT) samples (Figure 1 (B)). This fact agrees with other authors who reported that light-colored honeys (like rosemary) follow a Newtonian behavior (B .Abu-Jdayil *et al.*, 2002; L. Juszczak and T. Fortuna, 2006; P.A. Sopade *et al.*, 2003; S. Yanniotis *et al.*, 2006). However, no studies reporting neither the viscosity of rosemary honey nor the effect of US on the viscosity of honey have been published so far.
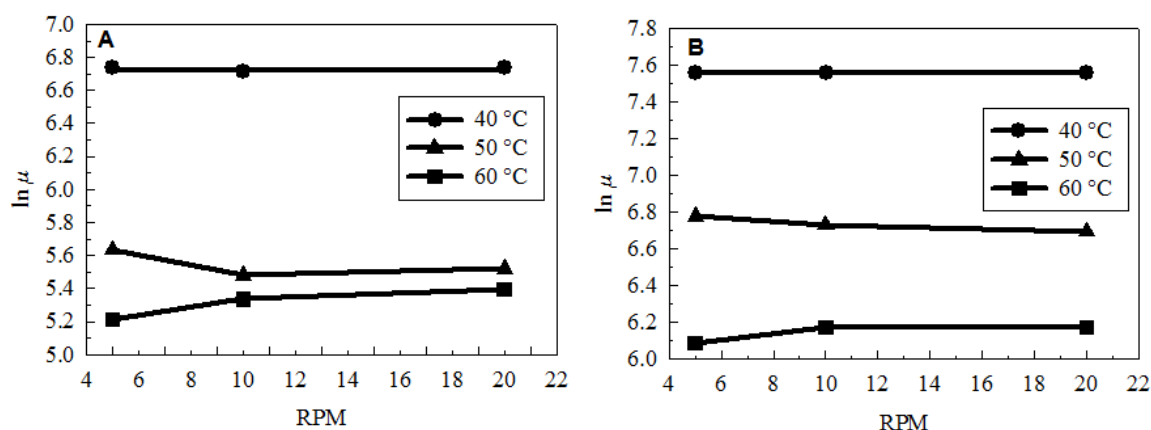
Figure 1. (A) Temperature effect on viscosity of US-treated samples for rosemary honey type. 40°C (●); 50°C (▲); 60°C (■). (B) Temperature effect on viscosity of HT samples for rosemary honey type 40°C (●); 50°C (▲); 60°C (■).

Table7 lists the data of the viscosity values obtained after the ultrasound-treated and heat-treated honey samples and the decrease of viscosity between these samples. The reduction in viscosity ranges from 16.5 % at 60 °C during 10 min up to 70 % after 40 min at 50 °C. The viscosity values of the heat-treated samples obtained in the present study are higher compared to the data (honey) cited in the literature. For example, at a temperature of 40 °C the maximum viscosity

attained by a sample of Australian honey (Yapanuyah) was 1.9 Pa s (P.A. Sopade *et al.*, 2003) and 3.5 Pa s of Polish Nectar honeydew (S. Yanniotis *et al.*, 2006), while the value reached in this study was 5.4 Pas. The same behaviour is observed for samples heated at 50 °C and 60 °C. This could be justified by the fact that the viscosity of honey not only depends on the water content but also on its origin, so its chemical constitution. S. Yanniotis *et al.* (2006) well-pointed in their work: P.A. Sopade *et al.* (2003) presented viscosity values that differed from those obtained in earlier investigations with the same honey varieties reported by B. Bhandari *et al.* (1999) both Australian honeys, which is due to the differences in the chemical constitution of the samples, i.e. the climatic, processing and storage conditions. Figure 2 (A)–(C) shows the honey flow behaviour. It can be seen the sharpest decline of viscosity occurs in the first 20-25 min, and that an US treatment falls much more in the honey consistency as the viscosity decreases considerably compared to the only HT samples. T-tests were used to analyze the relationship between the means of viscosity of the US and the HT and corroborated significant difference. These results clearly assert that using ultrasound waves the liquefaction become faster without increasing the temperature up to 50 °C, saving thus the energy consumption. This accomplishment also agrees with similar works from other authors like A. Thrasyvoulou *et al.* (1994) and B. d'Arcy (2007), who also processed honey with ultrasounds. The authors stated that honey became liquefied but they focused their works on the quality parameters of honey after being treated, but they did not present rheological measurements.

Table 7. Mean and standard deviation viscosity values and difference of values for the US-treated samples and HT samples.

Treatment time	US 40 °C (mPa s)	HT 40 °C (mPa s)	Difference (%)
10 min	3680±13	3240±56	-12.00±3.55
20 min	1900±28	2200±28	14.00±2.25
30 min	1393±30	1740±20	20.00±2.25
40 min	925±50	1637±11	43.51±3.55
60 min	833±23	1495±10	44.28±18.23
Treatment time	US 50 °C (mPa s)	HT 50 °C (mPa s)	Difference (%)
10 min	1520±80	1620±53	12.35±6.70
20 min	646±11	853±23	24.22±2.82
30 min	390±10	780±20	50.00±2.10
40 min	240±0	780±20	69.23±0.91
60 min	230±10	726±11	68.32±1.68
Treatment time	US 60 °C (mPa s)	HT 60 °C (mPa s)	Difference (%)
10 min	960±90	1150±55	16.33±10.17
20 min	400±20	533±20	25.00±5.42
30 min	313±20	400±23	21.75±7.79
40 min	246±11	400±0	38.50±3.17
60 min	206±18	333±11	38.20±6.68
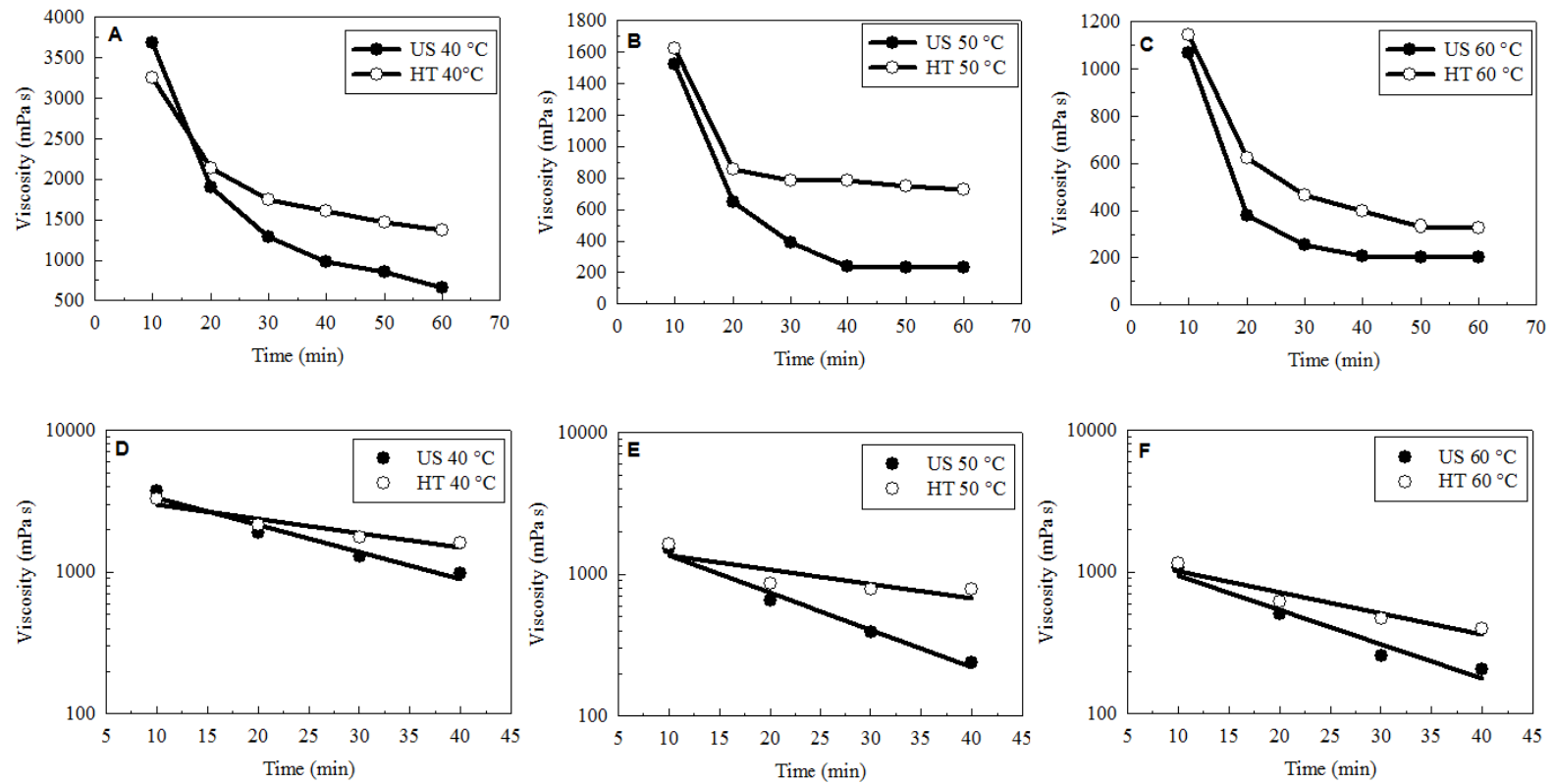
Figure 2. Viscosity behavior of US (●) treated samples and HT (○) samples at: (A) 40°C, (B) 50°C, and (C) 60°C. Viscosity exponential regression of US (●) treated samples and HT (○) at: (D) 40°C, (E) 50°C and (F) 60°C.

An Arrhenius-type model was used to study the flow behaviour of honey. First, the viscosity data was analyzed within time and temperature. By this analysis it was aimed to demonstrate the effectiveness of an ultrasound treatment, relative to heat treatment, for the liquefaction of honey. Second, the flow behaviour of honey was studied with respect to the temperature change at a constant shear rate (spindle speed) after a certain period of time, to ensure the incidence of the ultrasound treatment with the assistance of temperature.

Firstly, the flow behaviour was fitted to the equation:

$$\mu\ (t) = \mu_I \exp\ (-k\ t), \qquad\qquad\qquad\qquad \textit{Equation 2}$$

where μ is the viscosity (Pa s) after t minutes (treatment), μ_I the initial viscosity (Pa s) and k is the reaction rate constant (min^{-1}). Then, the dependence of k on temperature was described by an Arrhenius equation:

$$k\ _{(T)} = k_R \exp\ (-\varepsilon/RT), \qquad\qquad\qquad\qquad \textit{Equation 3}$$

where k_R is the pre-exponential factor (min$^{-1)}$, ε the activation energy (kJ g^{-1} mol^{-1}), R the gas constant (8.14 J kg^{-1} K^{-1}) and T the absolute temperature in Kelvin.

Figure 2 (D)–(F) are semi-logarithmic plots of the measurements shown in Firgure 2(A)–(C). The exponential regression was drawn taking the measurement of the first 40 min since this was the period in which a real exponential behaviour was observed. The fitting parameters and R^2 values are presented in Table 8 .Next, a linear relationship of ln $k(T)$ versus (1/T) was drawn to obtain the activation energy (Fig. 3(A)). The activation energy calculated was 20 kJ g^{-1} mol^{-1} for the US samples and 16 kJ g^{-1} mol^{-1} for the HT samples. Higher ε means that the honey viscosity is more sensitive to temperature change. Thus, Arrhenius model substantiate that US treated samples are prone to liquefy faster than the only HT samples.

Table 8. Equations calculated from an exponential regression by fitting the viscosity values (mPa s) versus time (min) at 40°C, 50°C and 60°C.

Sample	Equation	R^2	k_T	$\ln k_T$	E_a (kJ/mol)
40 °C US	$\mu = 5124e^{-0,044x}$	0.96	0.044	3.123	
50 °C US	$\mu = 2495.1e^{-0,06x}$	0.98	0.06	2.813	20.180
60 °C US	$\mu = 1653.9e^{-0,068x}$	0.96	0.07	2.659	

Sample	Equation	R^2	k_T	$\ln k_T$	E_a (kJ/mol)
40 °C HT	$\mu = 3739.3e^{-0,023x}$	0.90	0.023	3.772	
50 °C HT	$\mu = 2256.5e^{-0,029x}$	0.98	0.029	3.540	16.962
60 °C HT	$\mu = 1423.4e^{-0,034x}$	0.92	0.034	3.381	

The second objective was to determine the viscosity with respect to the temperature change at a constant shear rate (spindle speed) after a certain period of time. Thus, Arrhenius model was used to determine the effect of temperature on the viscosity:

$$\mu \ (T) = \mu_0 \ \exp \ (E_a /RT),$$

Equation 4

where μ is the viscosity (Pa s) after t minutes (treatment), μ_0 the constant, E_a the activation energy
(kJ g^{-1} mol $^{-1}$), R the gas constant (8.14 J kg $^{-1}$ K^{-1}) and T the absolute temperature (Kelvin).
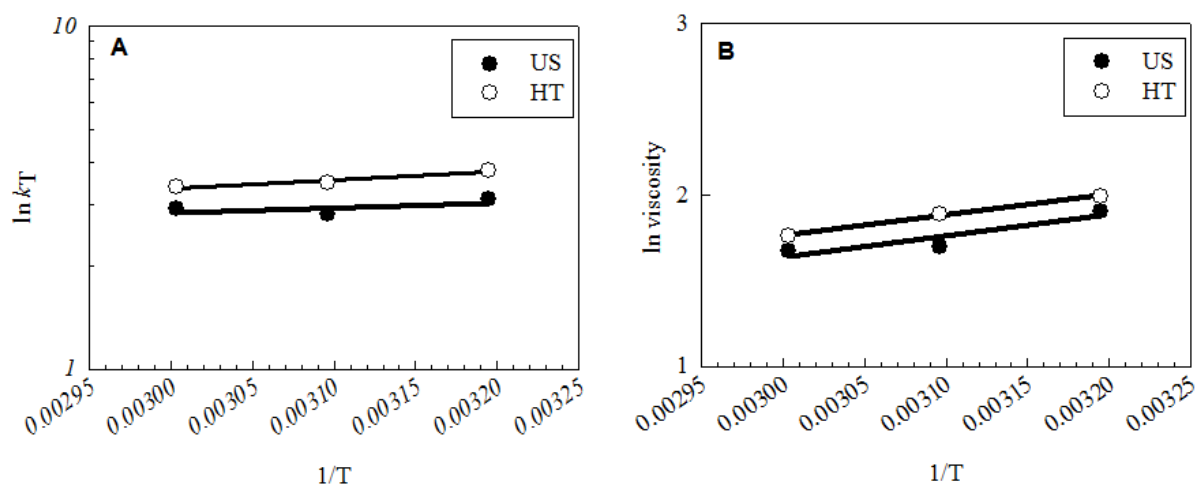
Figure 3. (A) Linear plot of ln k_T versus (1/T) for the US ($\bullet$) and HT ($\circ$) samples to obtain the E_a. (B) Linear plot of ln μ versus (1/T) for the US ($\bullet$) and HT ($\circ$) samples to obtain the E_a.

The viscosity was substantially reduced as the temperature was increased. This is of course expected because of the dependence of viscosity on temperature. Table 9 lists the *ln* of viscosity of both US and HT samples and Figure 3 (B) shows the logarithmic plot of *ln* l versus (1/T) from which we obtained the activation energy (E_a). The E_a calculated was 59 kJ g^{-1} mol^{-1} for the US samples and 64 kJ g^{-1} mol^{-1} for the HT samples. These results evince that even being treated at the same temperature after a certain period of treatment, ultrasound treated samples presented lower viscosity than only heat treated samples. These values are in the same range as those reported by P.A. Sopade *et al.* (2003), whose E_a values ranged from 66.31 to 103.70 kJ g^{-1} mol^{-1}. It should be noted that US samples presented lower activation energy than HT samples. Thus, results show that US treated samples are the least sensitive (lower E_a value); whereas HT samples are the most sensitive (highest E_a value) among the treatments examined. The viscosity behaviour is also a function of composition of sugars, moisture content, colloids and other components present in the honey (B. Abu- Jdayil *et al.*, 2002; B. Bhandari *et al.*, 1999). These factors were not individually considered in this study.

Table 9. Viscosity values after 60 min of treatments. US (●); HT(○).

Temperature	ln μ US sample	ln μ HT sample
40 °C	6.725	7.309
50 °C	5.438	6.587
60 °C	5.327	5.809

The sharpest decline of honey viscosity occurs in the first 20 minutes of treatment at 50°C. To compare the rate of liquefaction speed, that is the reduction in time of honey deliquescence, through the ultrasonic treatment compared to the only heat treatment, the time required to decrease the viscosity up to 10 % of its initial value was recorded. Both samples were treated at 50°C; the US samples were sonicated at 40 kHz while the HT samples were placed in the thermal bath without sonication.

As it can be seen in Table 10 both samples, the US and the HT, presented an initial viscosity value around 13000 mPa·s, however, it was required 9 minutes of ultrasound treatment to reach a final viscosity of 1266 mPa·s while it took 23 minutes for the only heat-treated ones. Figure 4 shows the liquefaction curves of US and HT samples, as it can be observed, the ultrasound treatment speeds up the liquefaction time. Results reported are the average of 3 determinations.

Table 10. Viscosity (mPa s) decrease in time of US and HT samples.

Time (min)	US (mPa s)	HT (mPa s)
0	12733± 25	12900±23
1	7890± 30	10586±20
3	5300±20	7000±31
5	3166±20	4833±22
6	2100±23	4680±20
9	1266±11	3166±25
11		2133±31
21		1433±32
23		1266±32
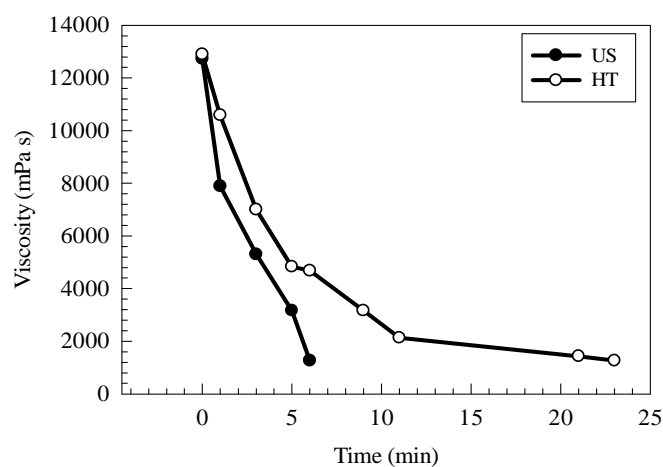
Figure 4. Liquefaction rate of US and HT samples.

4.3.2. Crystal observation

When honey is being heated, the sugar crystals redissolve to a liquid state, or as is attempted to demonstrate, an US treatment also liquefies the honey, having more effective influence in the de-crystallization as a means of reducing the crystal size, and thus improving the spreadability of the product. The microscope photographs gave an indication of the structure of the honey crystals in different honey. Picture 8 shows a representative picture of the crystal content of honey before being treated. It is presented as a complex structure of fine needle-shaped crystals; this is because there is so much sugar in honey relative to the water content. Glucose tends to precipitate out of solution and the solution changes to the more stable saturated state. For all the pictures taken, the microscope was graduated to 20 increases having a factor of 8. The camera used to take the pictures had a factor of 0.8. The total increases were 128. The crystal content was carried out taking many photos of which the most representative are shown below.

Pictures 9, 10 and 11 show the crystal content comparison between the heat-treated and the US-treated samples after applying their respective treatments.
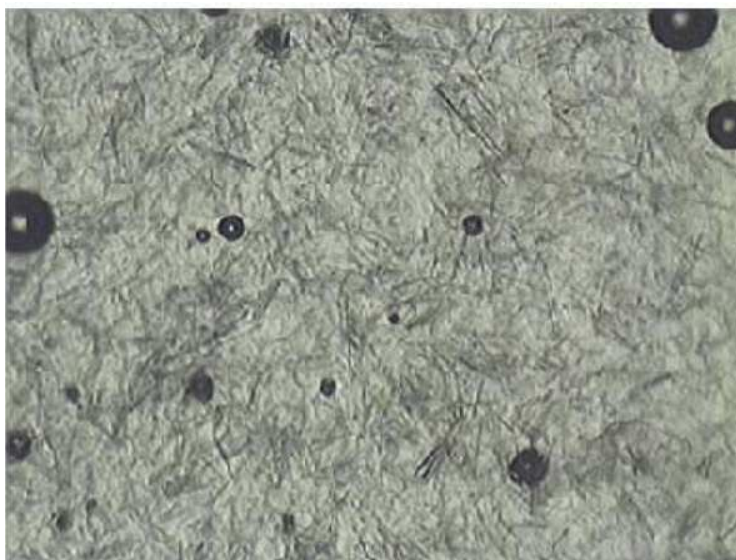
Picture 8. Control sample. Before being treated honey appears as network of needle-shaped crystals. Dark circles are air bubbles.

As Picture 9, 10 and 11 ((A), (B) and (C)) show, the higher temperature the lower crystal content. When honey is treated by ultrasounds or heated, the sugar crystals redissolve to a liquid state. Visual observation of the containers containing honey subjected to ultrasound and heat treatment indicated that the crystals were dissolved, and the honey was a clear transparent liquid.

Picture 9 (A) and (A1) shows the picture of a representative heat-treated sample at 40 °C during 20 min compared with a picture of an US-treated sample at 40 °C and sonicated during 20 min. It can be noticed that the US-treated honey presented a clearer appearance than the only HT sample. In addition, more needle crystal masses remained in the heat-treated honeys than were abided in the ultrasound-treated honeys. Nevertheless there still was some crystal clusters in the honey in both samples. It is worth mentioning the change of crystal structure; the initial honey presented clean sharp needle-shaped crystals while ultrasonicated and heat-treated samples showed irregular large pentagon shaped plate crystals too. B. D'Arcy (2007) reports that this is probably because the US rather than reducing the crystal size through breakup of the crystals, the crystal size is reduced through partial melting or dissolution of the D-glucose monohydrate crystals.

Picture 10 (B) and (B1) shows the picture of a representative heat-treated sample at 50 °C during 20 min compared with a picture of an US-treated sample at 50 °C and sonicated during 20 min. As it can be seen, there is severely less crystal content in the US-treated samples and no crystal clusters were observed as they were swooned. Just in the first 20 min of treatment, the needle-shaped crystals became melted notably.

Picture 11 (C) and (C1) shows a representative picture of a heat-treated sample at 60 °C during 20 min compared with a picture of an US-treated sample at 60 °C and sonicated during 20 min. The samples with the US bath treatment presented drastically clearer and transparent appearance than the only HT honey samples. The crystal content was reduced through melting or dissolution and crystals size were also considerably smaller. No needle crystal masses were observed in the US samples anymore.

These findings are consistent with those obtained by B. d'Arcy (2007) in his work, who also analyzed the crystal content of honey treated by an ultrasound probe. The author also found out that an ultrasound treatment delays D-glucose monohydrate crystallization more than does a heat treatment similar to that used by the honey industry. In addition, he reported to be a difference in the crystal formation process at the microscopic level in ultrasound-treated honey relative to that in heat-treated honey.

Thus, the ultrasound treatment will not only liquefy candied honey without the need for long exposure to high temperatures, but may make the liquefied honey more stable to subsequent crystallisation on storage.
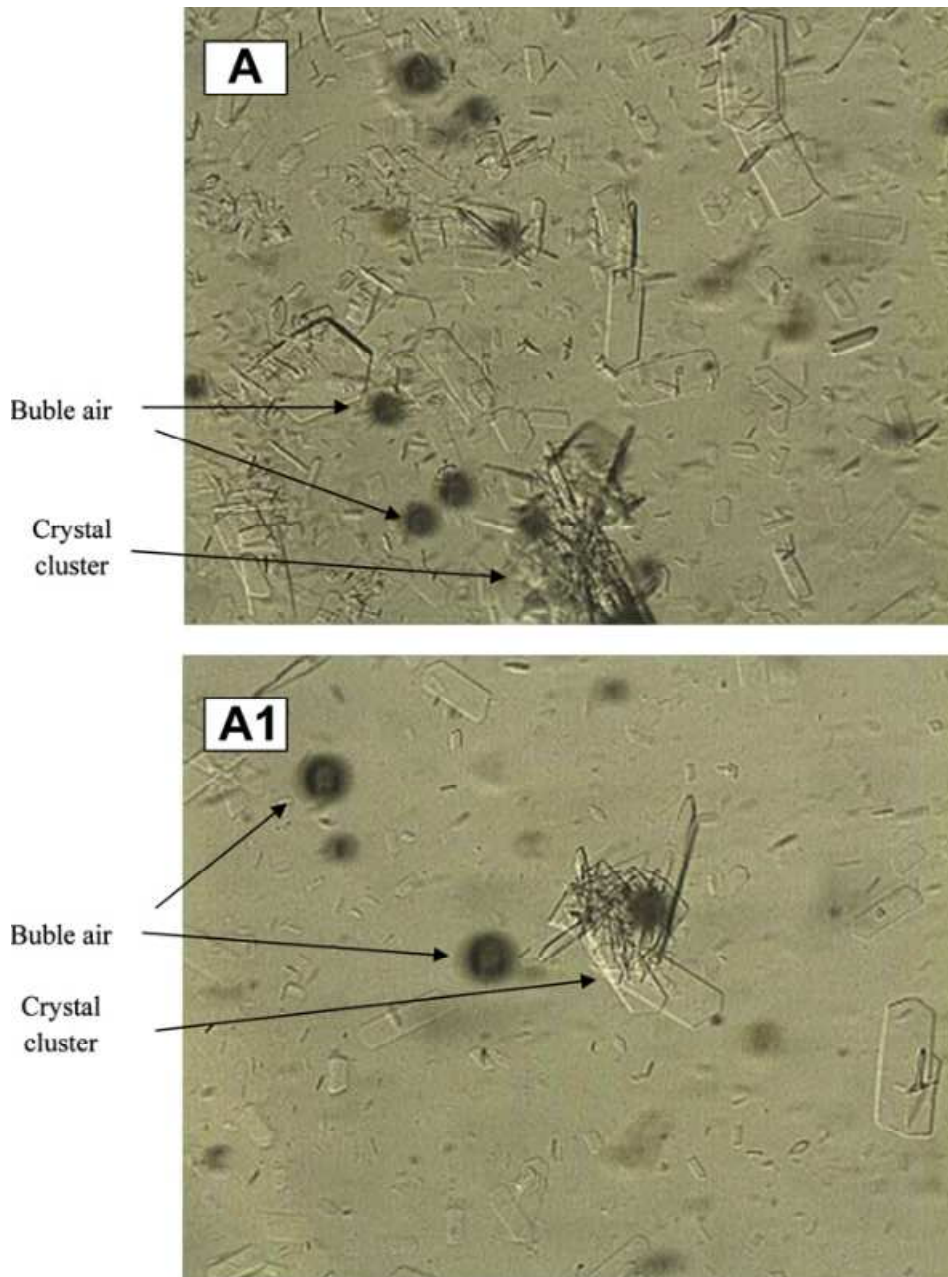
Picture 9. (A) 40 °C Heat-treated samples after 20 min of thermal treatment; (A1) 40 °C + US-treated samples after 20 min of treatment. As it can be observed, after just 20 min of treatment the US treated samples presented a decrease in number and size of crystals compared to the HT samples.
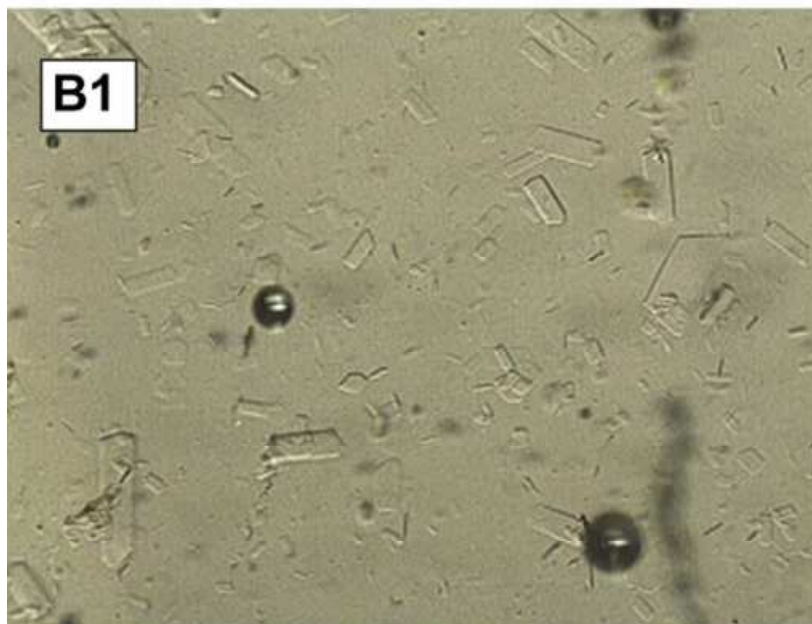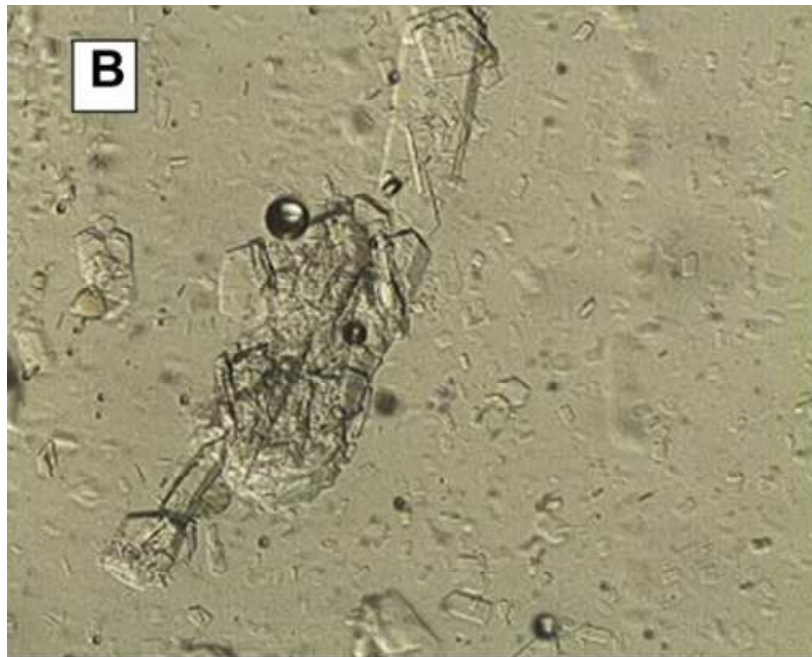
Picture 10. (B) 50 °C Heat-treated samples after 20 min of thermal treatment; (B1), 50 °C + US-treated samples after 20 min of treatment. The big sizes of crystals are not presented in the US treated samples.
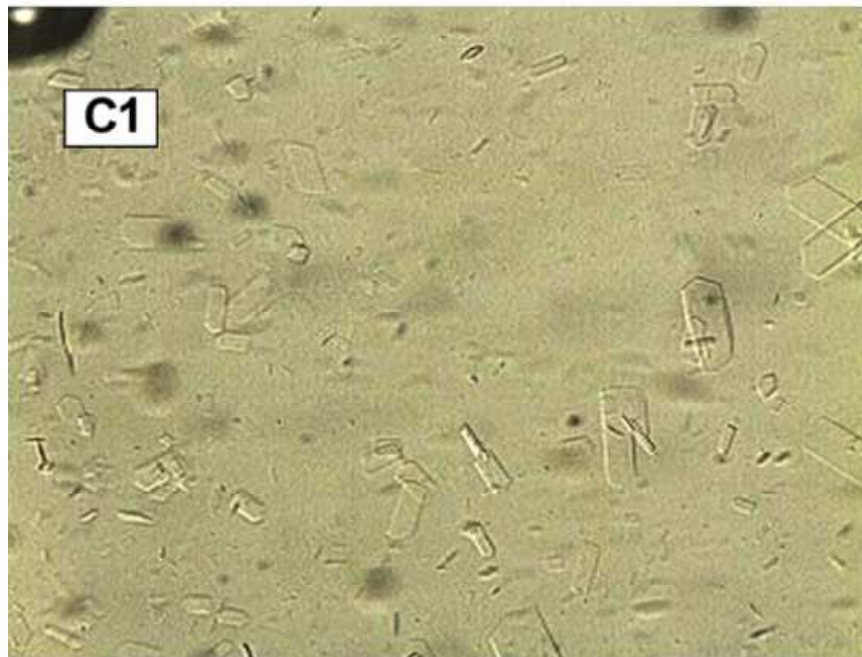
Picture 11. (C) 60 °C heat-treated samples after 20 min of thermal treatment; (C1), 60 °C + US-treated samples after 20 min of treatment. The crystal content of the ultrasound-treated samples presented a clearer and transparent honey than only heat-treated honey samples.

4.3.3. Storage behavior (re-crystallization)

The speed of honey to crystallize depends on the presence of catalysts, like seed crystals, pollen grains and pieces of beeswax in the honey. These minute particles serve as nuclei for crystallization. Raw honey contains bits of wax, pollen and propolis, and crystallizes faster. Honey that has been processed (e.g. heated and sonicated) will remain in its liquid form longer than raw honey due to the elimination of nuclei, which encourage the growth of glucose crystals.

Turbidity of honey increases with granulation, and an intensity increase in the absorbance at 660nm is considered a valid measure of determining the granulation extent (C.Lupano, 1997). That is, the lower the absorbance the clearer the honey.

The absorbance of honey at 660 nm was measured once a week during six month period (180 days). Honey samples were treated for 20, 40, and 60 minutes at 50°C and were kept at ambient temperature (approximately 25 °C) during measurements.

Typical results[2] obtained in this experiment are shown in Figure 5 for representative raw honey samples. As it can be observed, rosemary raw honey manifests a clear tendency to crystallize over the time. The curve of granulation of rosemary honey however, is less pronounced compared to those curves of Argentinean polyfloral honey reported previously by (C.Lupano, 1997) and M.S.Finola *et al.* (2007). Both authors recorded the absorbance of honey (660 nm) during storage and in both cases the absorbance exceeded 2 units in the first ten weeks (70 days).
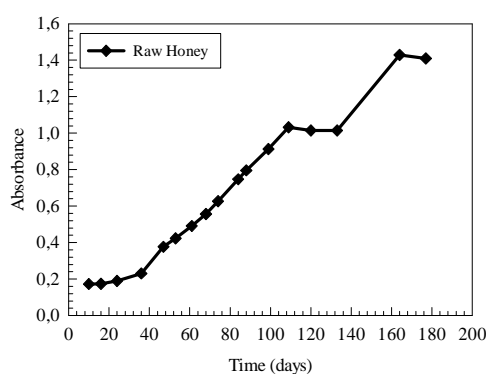
Figure 5. Absorbance at 660 nm of rosemary raw honey stored at room temperature as a function of the storage time.

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[2] Absorbance is presented in arbitrary units.

Figure 6(A) shows the absorbance of US honey samples as a function of storage time. The general behaviour observed is lower absorbance values at higher treatment times. Moreover, the absorbance increased very rapidly in samples sonicated during 20 min and more slowly in samples treated during 40 and 60 minutes. For the HT honey samples however, samples treated during 60 minutes presented initially the lower absorbance value but the increase was higher than those treated for 40 minutes (Figure 6 (B)). This fact indicates that ultrasounds liquefy better the honey and delays the re-crystallization than only a heat treatment, thereby having a longer shelf life.

Figure 7 (A, B, C) shows the tendency of re-crystallization between US and HT samples, in all cases the initial, increase (Δ Abs.) and final absorbance of the ultrasound-treated samples was lower than those only heat-treated. Table 11 shows the total increase of absorbance of raw, US and HT honey samples treated at 50°C for 20, 40 and 60 min. As it can be seen, raw honey presented the highest tendency of crystallization (increase of 1.24 units). For the treated samples, in all cases the increase of absorbance was higher in the HT samples than the US samples.

It is important to take into account that the time it will take the honey to crystallize depends mostly on the ratio of fructose to glucose. It has been observed that honeys with a high percentage of fructose remain liquid for a long time. Thus other ratios such as fructose/glucose or (glucose *minus* water)/fructose have been proposed (NHB, 2006). The use of these ratios to predict crystallization is possible only when comparing honeys which differ significantly in sugar composition. Hence, honey high in glucose sugar, with a low fructose to glucose ratio will crystallize more rapidly, such as alfalfa, cotton, dandelion, mesquite, mustard and rape (*brassica napus*). Honey with a higher fructose to glucose ratio (containing less than 30% glucose) crystallizes quite slowly and can stay liquid for several years without special treatment, for example, robinia (black locust), sage, longan and tupelo, (K. Flottum, 2009). Rosemary honey has an average concentration of 35% fructose and 31% of glucose (C. Pérez-Arquillué *et al.*, 1999; R. Mateo and F. Bosch-Reig, 1997). Therefore, rosemary honey in question is commonly classified as half prone to crystallization.
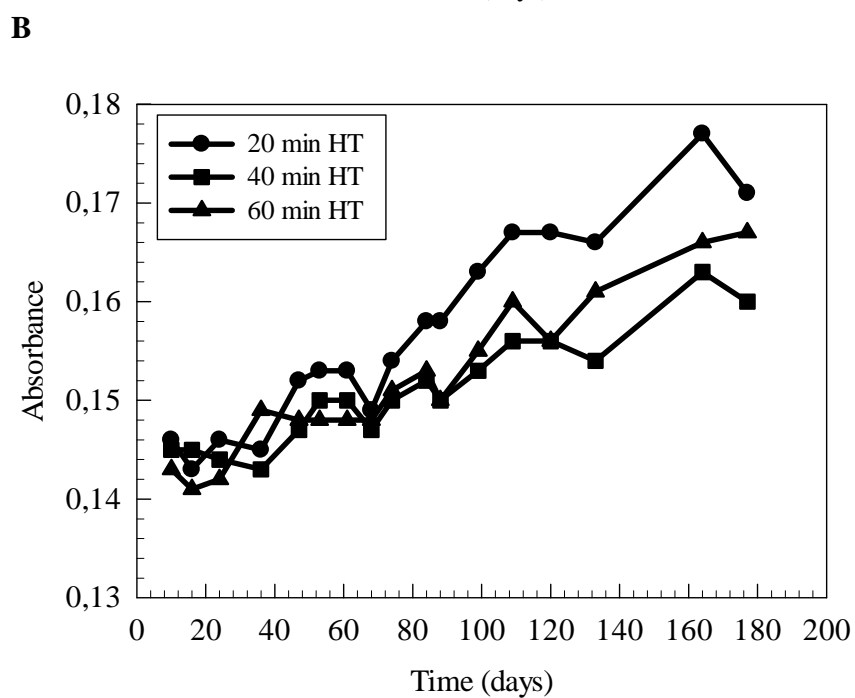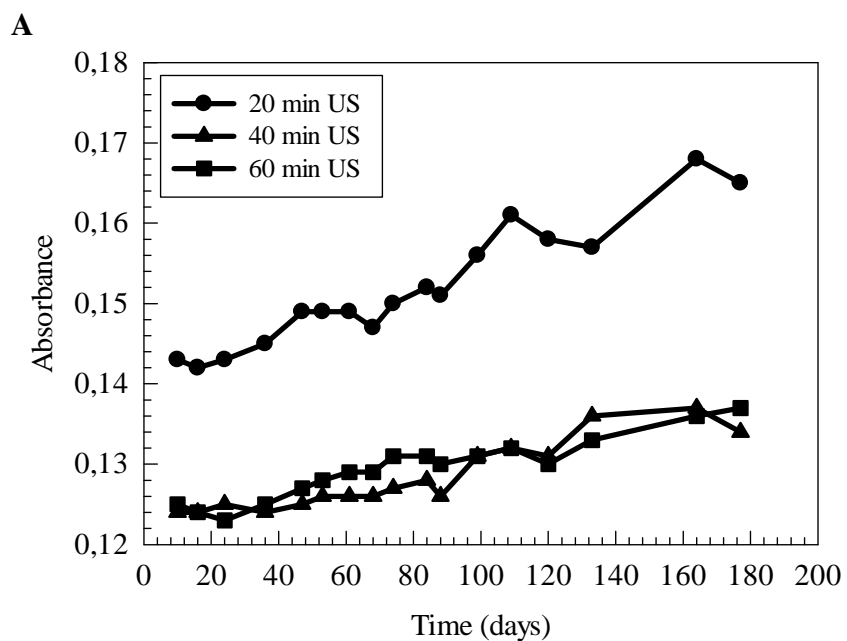
Figure 6. (A) Ultrasound-treated samples at 50 °C for 20 min (●), 40 min (▲) and 60 min (■). **(B) Heat-treated samples at 50 °C for 20 min (●), 40 min (▲) and 60 min (■) as function of storage time.**
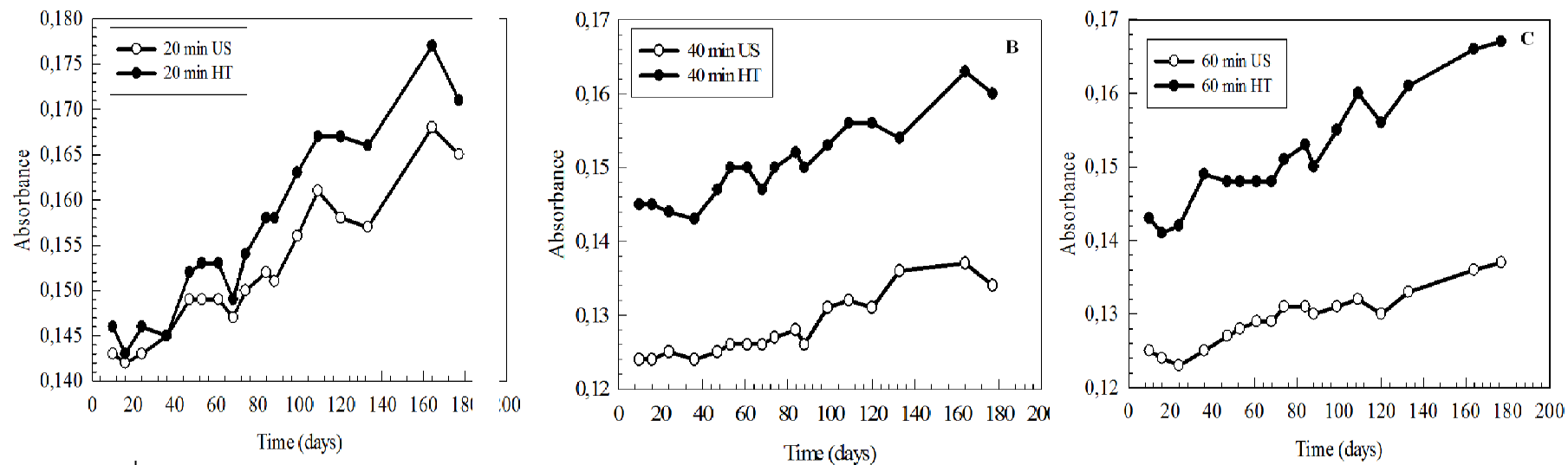
Figure 7 . Absorbance at 660 nm of US (○) and HT (●) honey samples treated at 50 °C for 20 min (A), 40 min (B) and 60 min (C) and then stored at room temperature as a function of the storage time.

Table 11. Initial, final and increase (Δ) of absorbance of raw, ultrasound (US) and heat-treated (HT) samples.

		initial Abs.	final Abs.	Δ Abs.
	Raw Honey	0,173	1,409	1,236
20 min	US honey	0,143	0,146	0,003
	HT honey	0,165	0,171	0,006
40 min	US honey	0,124	0,134	0,010
	HT honey	0,145	0,160	0,015
60 min	US honey	0,120	0,132	0,012
	HT honey	0,140	0,164	0,024

The storage temperature also has a big effect. Honey crystallization is most rapid around 10-15 ºC. At temperature below 10 ºC the crystallization is slowed down. Low temperature increases the viscosity of honey (honey is thicker when cool), and this retards the formation and diffusion of crystals. Honey resists crystallization best at higher temperatures more than 25 ºC. When the temperature is 40 ºC the crystals dissolve. Temperature above 40 ºC will damage the properties of honey over time (E. Crane, 1983; E. Tosi *et al.*, 2004; K. Flottum, 2009; K. Hamdan, 2012).

In order to verify whether there was an increase of the total crystal mass during long-period storage, samples treated in point 1.2.1. Sample treatment (also refer to viscosity section) were stored at 4 °C for 2 years. Hence, storing the honey at 4 °C is supposed to result in homogeneous and fine-grained crystals (E. Venir *et al.* (2010). The more rapid honey crystallizes, the finer the texture will be. Crystallized honey tends to set a lighter/paler colour than when liquid (K. Hamdan, 2012). This is due to the fact that glucose sugar tends to separate out in dehydrating crystals form, and that glucose crystals are naturally pure white.

Picture 12 and 13 show the pictures of US and HT samples two years after liquefaction. A comparison of the two samples reveals again the incidence of US in honey liquefaction and its stability to remain liquid, since in all cases the US samples took longer to re-crystallize.

From Picture 2 it can be observed how HT samples at 40 °C completely crystallized, while the US at 40 °C did not fully crystallize. It is interesting to note that crystallization started at the bottom of the beakers but also occurred in the surface, covering the entire area. For samples treated at 50 °C, heat-treated samples partially crystallized along the container, while US samples did not form into crystals, but presented minute nuclei particles of crystallization. Heat-treated samples at 60 °C did not assume crystalline structure but there was some granulated crystal particles. For the US samples at 60 °C the granulated crystal particles observed were markedly less than the HT samples.

Picture 12. (A) Heat-treated (HT) and Ultrasound (US) treated samples treated at 40°C. (B). HT and US samples treated at 50 °C. (C) HT and US samples treated at 60°C. All samples were stored at 4 °C for two years after treatment.
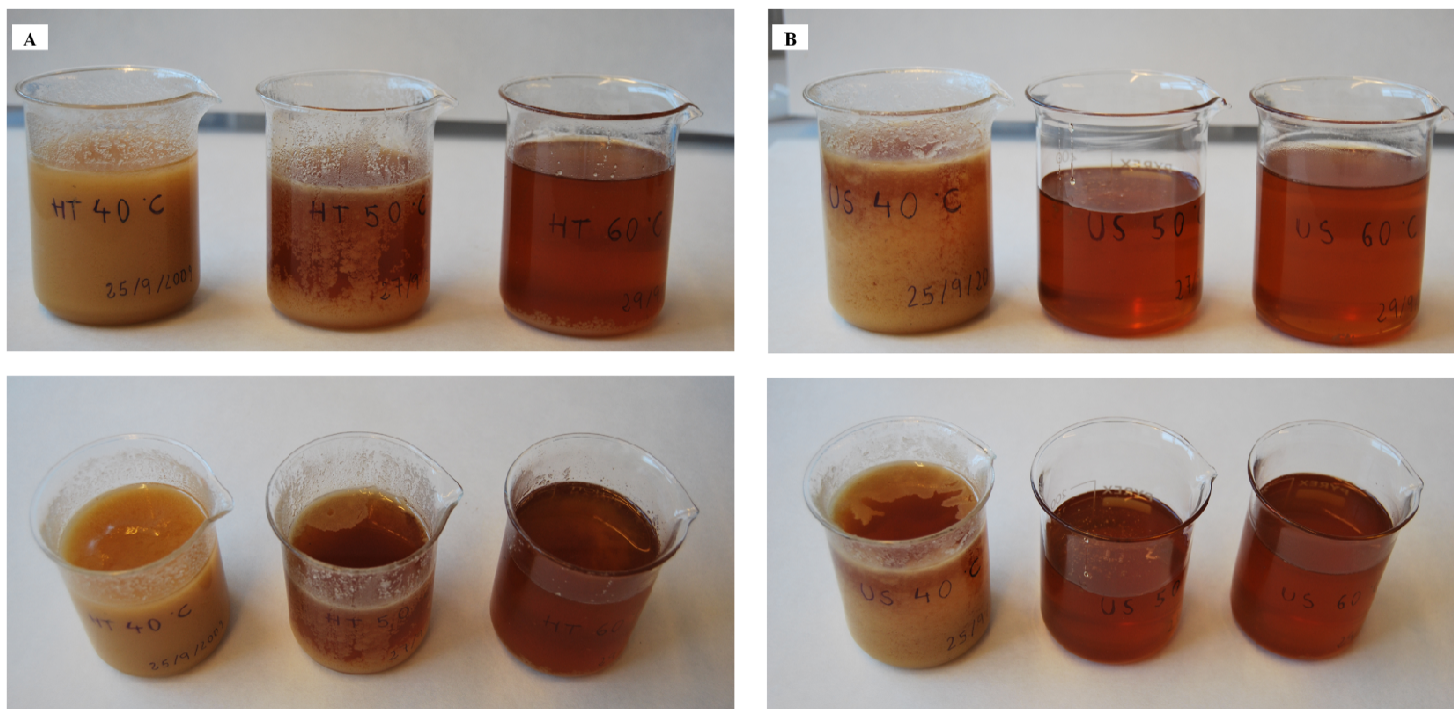
Picture 13. (A) Heat-treated (HT) samples treated at 40, 50 and 60 °C and stored at 4°C for two years. (B) Ultrasound-treated (US) samples treated at 40, 50 and 60 °C and stored at 4°C for two years.

The above experiment results show that an ultrasound treatment at 50 °C is effective to hinder the crystallization and therefore it is not require a higher temperature treatment. This facts is observed at both the microscopic level (in a drop of honey) and in bulk samples.

4.3.4. Differential scanning calorimetry (DSC)

The DSC operates on the principle of thermal transition. There are two aluminium pans, one is the reference pan and the other is the sample pan, and each pan sits on a heater. The reference pan was kept empty and about 5-8 mg of honey was weighed into a second aluminium sample pan and sealed with a lid. Heat flow was calibrated using Indium standard at the scan rate of 10 °C per min. These two pans were heated at the same rate according to a given method: +25 °C to -100 °C to +250 °C at the rate of 10 °C/min.

The DSC produces a graphic output of temperature versus heat flow. Glass transitions usually occur as the temperature (Tg) of an amorphous solid is increased. These transitions appear as a step in the baseline of the recorded DSC signal. This is due to the sample undergoing a change in heat capacity; no formal phase change occur (G. Höhne *et al.* 2003).

Glass transition temperature is extremely important from a practical perspective; for many materials it determines the highest use temperature, while at the same time it defines the lowest possible processing temperature (J. D. Menczel and R. B. Prime, 2009). As the temperature increases, an amorphous solid will become less viscous. At some point the molecules may obtain enough freedom of motion to spontaneously arrange themselves into a crystalline form. This is known as the crystallization temperature (Tc) (J. D. Menczel and R. B. Prime, 2009). This transition from amorphous solid to crystalline solid is an exothermic process, and results in a peak in the DSC signal. As the
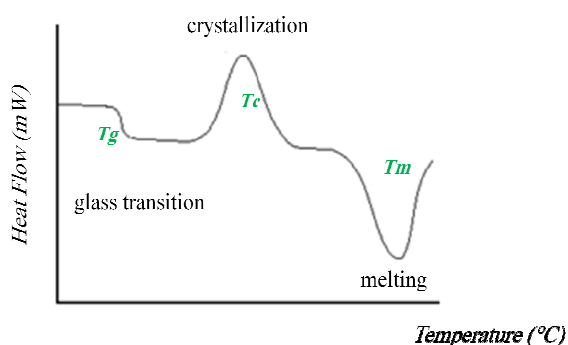
Picture 14. Schematic DSC plot of several common features

temperature increases the sample eventually reaches its melting temperature (Tm). The melting process results in an endothermic peak in the DSC curve.

The calorimetric enthalpy value for the phase transition is then determined by integrating the area under the peak of the transition (ΔH). Specific heat capacity (Cp) can also be measured by DSC plots, since the signal is closely proportional to the heat capacity of the sample. The specific heat capacity indicates how much heat is needed to raise the temperature of 1 g of the material by 1 °C. Both, ΔH and Cp can be calculated using the standard software of the DSC (STAR® thermal analysis system version 10.00).

Picture 14 presents a schematic DSC curve showing the appearance of several common features mentioned previously.

Honey samples were treated at 50 °C for 2 h in a water bath (HT samples) and sonicated in an ultrasonic bath (US samples) at the same conditions.

Typical DSC curves recorded during thermal scanning of honey samples are shown in Figure 8. As it can be seen, the peak of Tc was not observed in the DSC plot throughout the temperature range investigated. This is probably because the phenomenon of honey granulation (crystallization) consists of two processes; the formation of the crystals and their gradual growth (C.Lupano, 1997) hence, at the scanning rate of 10 °C/min there was no enough time for crystallization to occur.

The inflection Tg of Raw, US and HT honey samples were observed at -42.03 °C, -42,70 °C and -41.68 °C, respectively, which was in good agreement with the literature values from -33.64 to -51.14 °C (J. Ahmed *et al.*, 2007)). The major sugars present in honey are fructose and glucose (S. Ouchemoukh *et al.*, 2007) which are contributed for glass transition of honey samples; however, the sugar ratio depends on floral source and environmental conditions that finally govern honey Tg (J. Ahmed *et al.*, 2007). In addition, Tg varies widely for a specific honey as it depends on many factors like sample preparation and size, heating/cooling rate, sample holding time, moisture content, etc.
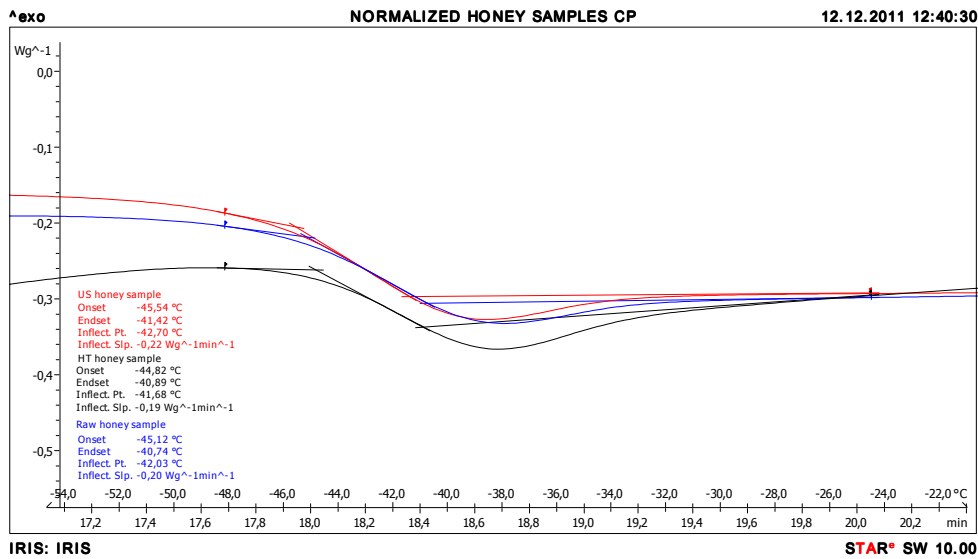
Figure 8. DSC plot. Glass transition temperatures *Tg*. (Red) Ultrasound-treated sample *Tg* of -42,70 °C. (Black) Raw Honey sample *Tg* of -42.03 °C (Blue) Heat-treated sample *Tg* of -41.68 °C.
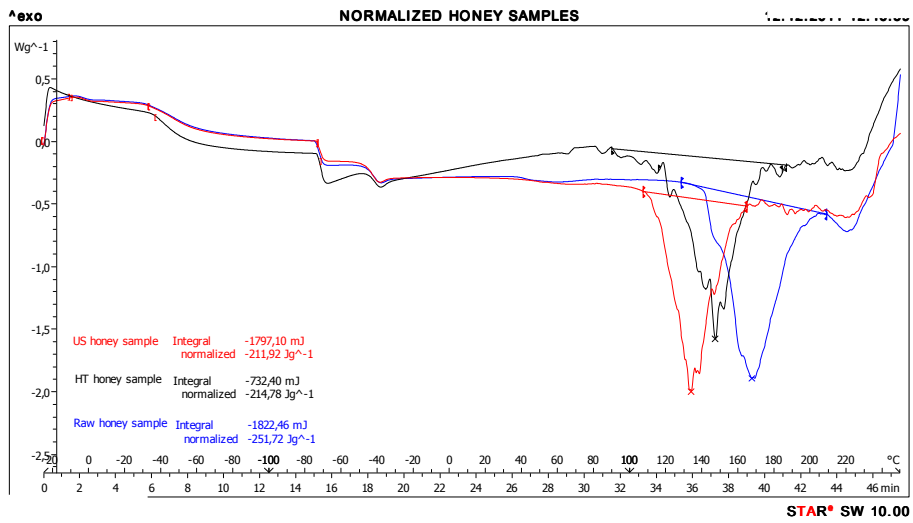
Figure 9. DSC plot. Specific heat capacity (*Cp*). (Red) Ultrasound-treated sample *Cp* 2.37 J/g °C (Black) Raw Honey sample *Cp* of 2.72 J/ g °C (Blue) Heat-treated sample *Cp* of 2.62 J/g °C.

The specific heat capacity (Cp) of the present study for raw honey was 2.72 J/ g °C, which is in the range of other authors (2.26 J/ g °C, J.White (1975), 1.97-3.07 J/ g °C (P.A. Sopade *et al.* (2005)).

The specific heat capacity of the US samples was 2.37 J/g °C and 2.62 J/g °C for the HT samples. These slightly differences indicate that honey is more sensitive to a temperature change (requires less energy) once treated, and that the combination of heat and ultrasounds falls more in the honey consistency.

On the other hand, the DSC plot shows the small energy changes that occur, as matter transitions from a solid to a liquid crystal. Transition enthalpy is the amount of energy absorbed to take place the phase change, that is, change from the crystalline structure to the "rubber-like" state of honey. Thus, the more crystals in honey, the higher value of enthalpy. When the crystals in the crystallized honey dissolve, heat is absorbed. After the temperature of sample pan reaches the dissolution temperature of the crystals, the temperature will not increase until all the crystals are dissolved. Therefore, the heater under the sample pan has to heat the sample to dissolve the crystals, as well as increase the temperature at the same rate as the reference pan. This extra heat flow during dissolution of the crystals is shown as a peak in the DSC plot. The melting point of honey commonly occurs at 170-240 °C range temperature (J. Ahmed *et al.*, 2007; B. d'Arcy, 2007). Raw honey presented the highest enthalpy of 251.72 J/g followed by the heat-treated (214.78 J/g) and the ultrasound-treated sample (211.92 J/g). Thus, the effect of ultrasounds on honey liquefaction (glucose crystal dissolution) is much incident than an only a heat treatment. This fact is interesting from an industrial point of view, as it will reduce the energy costs.

1.3.5. Location of the highest intensity inside the ultrasonic bath

Indirect application of ultrasounds is performed using an ultrasonication bath. The ultrasonic wave needs first to cross the liquid inside the ultrasonic device and then to cross the wall of the sample container. The ultrasonic intensity distribution inside the ultrasonic bath however, could not be homogeneous. Therefore, it is recommended to locate the position that has the highest intensity of sonication in the bath (J.L. Capelo-Martínez, 2008). The aluminium foil test is considered a valid method for this purpose.

The ultrasound bath used in throughout this thesis worked with only one frequency, 40 kHz, with 3 power intensities and supplied with temperature control.

A series of aluminium foil sheets were placed horizontally (covering entirely the bottom, Picture 15 second row) and vertically (Picture 15 first row), the most intense zones of sonication inside the bath were quite accurately identified. As consequence of cavitation the aluminium foils were perforated after 1 minute of sonication. The maximum perforations occurred at maximum intensities (Picture 15 (A). It can be observed that ultrasonic waves were spread evenly far and wide the bath, having a homogenous effect across the surface. However, it should be noted that the effect was accentuated in the centre of the bath.

Thence, the US samples will be located at the point where the maximum effect was achieved.
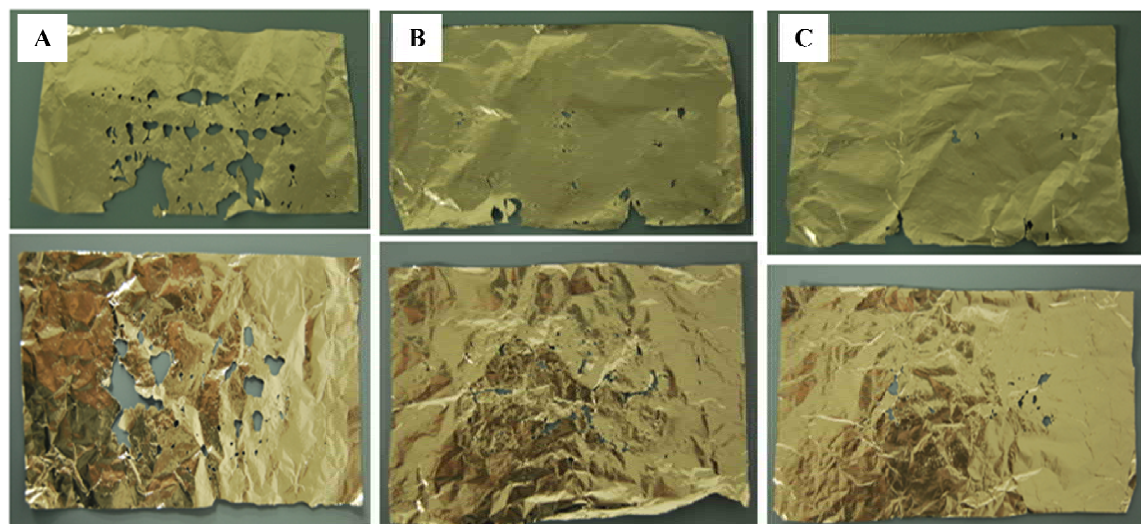
Picture 15. Ultrasound effect on aluminium sheets. (A) Power intensity 3. (B) Power 2, (B) Power 1. All powers tested during 1 minute.

4.4.   Conclusions

1. Different combinations of ultrasound treatment (temperature and duration) can be used to achieve the main objective of honey deliquescence. It was found that at a constant frequency of 40 kHz, an increase in sonication time or the level increased the amount of crystal disintegration. Ultrasound treatment can be effectively used for thermal processing of honey, as it speeds up the liquefaction of honey (i.e. 9 min of US treatment *vs.* 23 min of only HT) in a temperature range of 40–50 °C. This indicates that honey can be liquefied by US waves without the need to increase the temperature up to 50 °C or even higher.

2. The analysis substantiated that US treated samples are prone to liquefy faster than the only HT samples as the activation energy of the US treated sample was higher (20 kJ g^{-1} mol^{-1}) than the HT samples (16 kJ g^{-1} mol^{-1}), once treated at the same temperature. The E_a obtained from Arrhenius model of the US samples was lower than the HT samples, which indicate that US-treated samples are the least sensitive (lower E_a value), where as HT samples are the most sensitive (highest E_a value) among the treatments examined.

3. The crystal content of ultrasound-treated samples presented a clearer and transparent honey than only heat-treated honey samples. The amount and size of the crystals were also the smallest.

4. Ultrasound-treated samples presented lower absorbance values in respect the only HT samples, which mean honey is more clear, limpid and translucent. Moreover the increase of absorbance over time (6 month period) was lower in the US samples. This fact indicates that ultrasounds liquefy better the honey and delays the re-crystallization than only a heat treatment, thereby having a longer shelf life.

5. For long-period storage (i.e. 2 years) at 4 °C, US samples took longer to re-crystallize, and at 50 °C no crystallization was observed in the US samples. These findings indicate that an ultrasound treatment at 50 °C is effective to hinder the crystallization and therefore it is not require a higher temperature treatment.

6. The specific heat capacity calculated from the DSC plot of the US samples was 2.37 J/g °C and 2.62 J/g °C for the HT samples. The slightly differences indicate that honey is more sensitive to a temperature change (requires less energy) once treated, and that the combination of heat and ultrasounds falls more in the honey consistency.

7. The enthalpy calculated of the sonicated sample was lower (211.92 J/g) than the only heat-treated (214.78 J/g), which in turn means that an ultrasound treatment will be more effective on the glucose crystal dissolution.

<p align="center">❈❈❈❈❈❈❈❈</p>

The results of this work open up the possibility of further investigation in many directions. The next work of the investigation is going to be the study of the effect of the ultrasound treatment on honey main quality parameters.

5. CHAPTER 2: ULTRASOUNDS ON HONEY QUALITY PARAMETERS

5.1. Introduction

Honey distinctive characteristics are not due to its stable major compounds, which can be found in any other sweet product such as sugar, molasses, syrup and marmalade, but to its multitude of minor components originated from the nectar and the bees themselves (E. Tosi *et al.*, 2004). The geomorphology and the diversity of flora, gives the opportunity to bees (*Apis mellifera*) of producing a wide variety of honeys form coniferous and citrus trees, thyme or other floral origin which gives to the final product those special sensorial properties.

Honey is commonly **heated when processed in order to liquefy it, dissolve crystals and inhibit microbial growth**. However, many of substances which give honey its specific aroma, flavour and some of its biological activity are unstable over time and thermolabile. Therefore, **heating may affect negatively the quality of honey modifying its essential composition**.

The quality of honey is mainly determined by its physicochemical, sensorial and microbiological characteristics.

Internationally, honey quality criteria are specified in Regulatory Standards, compiled in a Codex Alimentarius (CA) standard. In Europe, EC Directive 2001/110 sets the honey standards for commercialization of European honey. Both standards CA and EC are similar. The CA is more detailed, containing references to quality factors such as heavy metals, pesticides and adulteration. In Spain, Official Spanish state Bulletin (BOE 1986/15960) sets the quality standards (same as CA and EC) as well as the recommended classical methodology for determination of quality factors.

The two most important and limiting parameters of honey quality are hydroxymethylfurfural (HMF) content and diastase activity (DN). In fact, these two parameters are used as indicators of honey **overheating** or freshness (E. Tosi *et al.*, 2004; B. d'Arcy, 2007; I.Turhan *et al.,* 2008).

The Codex Alimentarius (2001), EC Directive 2001/110, International Honey Commission (2002) and the Official Spanish State Bulletin (2003) set the maximum concentration of **hydroxymethylfurfural (HMF) to 40 mg/kg** for honey from non-tropical regions and 80 mg/kg for honey from tropical regions. **The diastase activity must not be less than or equal to 8**,

expressed as diastase number (DN). However, in the UE Directive, the quality criteria of HMF content and diastase activity are indented for application by commercial partners and governments, while according to the CA standard they are only for voluntary application between trade partners.

The two principal sugars in honey are fructose and glucose, while sucrose is found in lower quantities. Besides, about 25 different sugars have been detected (E. Crane, 1983; J. White, 1975; S. Bogdanov, 2002). The content of fructose and glucose in honey varies from one type of honey to the other. Generally, fructose ranges from 30- 44 %, glucose from 25- 40 % and sucrose 0.5-2 %. The carbohydrates found in honey have the ability to improve the intensity of desirable flavours and reduce the intensity of others. Honey enhances sweetness intensity, decreases sourness, decreases the bitterness intensity and increases the acceptability of savoury products by modifying saltiness perception (National Honey Board (NHB), 2006). In fact, since fructose predominates in honey it tends to make it taste slightly sweeter than sugar. On the average, honey is 1 to 1.5 times sweeter (on a dry weight basis) than sugar (NHB, 2006).

In respect to reducing sugars (fructose and glucose), all Regulatory Standards impose reducing sugars ≥ 60 g/100 g, and non-reducing sugars (apparent sucrose) are set to be ≥ 5 g/100 g for the majority of honeys.

The sugar composition is important since it will also influence the tendency of honey to crystallize, its rheological behaviour and structure. The fructose/glucose ratio is the main reason that leads to crystallization of honey, and the relative percentage of each determines whether it crystallizes rapidly or slowly.

Honey's water activity varies between 0.5 (16% moisture) and 0.6 (18.3% moisture) in the 4-37 °C temperature range, and hence inhibit any microbial growth. Water activity also influences the crystallization of honey.

Many works have been focused to determine the most common properties of the floral honey such as HMF, diastase activity, water content, total acidity, and mineral elements and examined the effects of a thermal treatment on those parameters during overheating at medium (30-55 °C) and high (70-100 °C) temperatures (E. Tosi *et al.*, 2004; B. Fallico *et al.*, 2004; I.Turhan *et al.*, 2008). However, only a few works investigated the effect of ultrasounds on honey quality (refer to section 1.4.4. in Literature Review), and so far, no works have investigated the effect of using

a treatment consisting of high-power low-frequency (40 kHz) ultrasound bath on main honey quality parameters when exposed to a treatment process for liquefying crystallized honey.

Therefore, this chapter aimed at evaluating the effect of ultrasound treatments on honey main **quality parameters** on:

- **HMF formation** content after applying an US treatment.
- **Diastase activity** of ultrasonicated honey.
- **Sugar composition**; fructose, glucose and sucrose of US-treated honey.
- **Water activity** of the ultrasonicated samples.

Additionally, a **sensory evaluation** consisting of a Paired Comparison Preference Test will be performed by a consumer panel to estimate their preference choice when an US and a HT sample are presented at once. That is, to evaluate whether an ultrasound treatment affects to the sensorial properties of honey.

5.2. Material and Methods

5.2.1. Determination of Hidroxymethylfurfural (HMF) content in honey by HPLC

As described previously S.Ajlouni and P. Sujirapinyokul (2009) and I. Turhan *et al.* (2008) a honey sample (5 g) was dissolved with a magnetic stirrer in 25 ml Milli-Q water and transferred to a 50-ml volumetric flask. The solution was filtered through a 0.2 µm membrane filter before injection on the HPLC (Waters HPLC, model Alliance) for HMF analysis.

The column used was a Sunfire C-18, stainless steel column (Waters, 4.6 x 150mm; film thickness 5 µm), operated at 25 °C along with a C18 guard column. The mobile phase was water/ methanol (HPLC-grade) (80/20, v/v), and the flow rate 0.75 ml/min with an injection volume of 20 µl. Serial standard solutions of HMF (Sigma, Spain) from 1 to 20 mg/l was made in Milli-Q water, to generate a calibration curve at 280 nm (Waters, UV detector 2489).

Calculation of HMF formation kinetics

The kinetic data analysis will be performed according to I. Turhan *et al.* (2008) as described:

$$C\ (t) = C_0\ \exp(-\ k_T\ t)$$

<div align="right">*Equation 5*</div>

where C_0 is the total initial concentration of HMF (mg kg^{-1}), k_T is the reaction rate constant (min^{-1}), C is HMF concentration after t minutes heating at a given temperature (mg kg^{-1}), and *t* is the isothermal heating time.

The dependence of k_T on temperature will be described by the Arrhenius model:

$$k_T = k_R \exp(-E_a\ /RT)$$

<div align="right">*Equation 6*</div>

where k_R is the pre-exponential factor (min^{-1}), E_a is the activation energy (kJ mol^{-1}) and R is the universal gas constant (0.008314 kJ mol^{-1} K^{-1}). A linear plot of lnk_T versus (1/T) was drawn to obtain the activation energy.

5.2.2. Evaluation of diastase activity (DN)

As described at the Official State Bulletin (BOE 1986/15960) the diastase activity was measured using a buffered solution of soluble starch and honey which was incubated in a special glass test tube in a thermostatic bath until the endpoint was determined photometrically (Nicolet Evolution e 300, Thermo Electron corporation, England).

The unit of diastase activity, the Gothe unit, is defined as that amount of enzyme which will convert 0.01 g of starch to the prescribed end-point in one hour at 40 °C under the conditions of test. Results are expressed in Gothe units (or Schade units) per gram of honey.

A standard solution of starch, capable of developing, with iodine, a color in a defined range of intensity, is acted upon by the enzyme in the sample under standard conditions. The diminution in the blue color is measured at intervals. A plot of absorbance against time is used to determine the time t_x required to reach the specified absorbance, 0.235. The diastase number is calculated as 300 divided by t_x, provided the method is followed precisely. This method is based on the original work of J. E. Schade *et al.* (1958) as presented by the Codex Alimentarius method (CODEX STAN 12-1981) and the Spanish Official State Bulletin (BOE 1049/2003) for honey. Picture 16 shows a scheme of the steps followed in the laboratory for the DN determination.

Reagents required

The reagents used in this method were:

- Sodium chloride solution: 2.9 g of sodium chloride (Panreac, Barcelona, Spain) dissolved in water and dilute to 100 ml.
- Acetate buffer solution (pH 5.3): 43.5 g of sodium acetate tri-hydrated (CH$_3$-COONa·3 H$_2$O) (Panreac) dissolved in water, pH adjusted to 5.3 with 5 ml of glacial acetic acid (Panreac) and diluted to 250 ml with water.
- Starch solution:
    a. Determination of starch dry weight :

Two grams of air dry soluble starch (Panreac) were spread in an aluminium tray with a lid and were dried for 90 min at 130 °C. Then, the tray was cooled at room temperature for 1 h in desiccators and was re-weighed.

b. Preparation of starch solution:

Three grams of starch were weighed into a 250 ml conical flask and 90 ml of distilled water was added and mixed through a magnetic stirrer during 10 min. The solution was then boiled and transferred to a 100 ml volumetric flask. Then it was cooled in running water. (This is the stock solution).

- Iodine solution (dilute): 20 g of potassium iodide (Panreac) dissolved in water and 2 ml of iodine stock solution was added. Then it was diluted to 500 ml.

1. *Sample Determination*

Ten grams of honey were weighed into a beaker and dissolved in 15 ml of distilled water and 5 ml of acetate buffer. The solution was transferred quantitatively to a 50 ml volumetric flask containing 3 ml of sodium chloride solution and it was adjusted to mark with distilled water (sample solution).

2. *Calibration of the Starch Solution / Adjustment of Blue Value*

This procedure was carried out to determine the amount of water that had to be added to the reaction mixture so that the absorbance range of the iodine starch solution would be 0.745 to 0.770.

Different volumes of 20, 21, 22, 23, 24 and 25 ml of water and 5 ml of dilute iodine solution were pipetted into 6 Falcon flasks. Then, 0.5 ml of a mixture containing 10 ml of water and 5 ml of starch solution was added to the first test tube; it was mixed well by agitating and immediately read the absorbance at 660 nm against a water blank in a 1 cm cell. It was proceed in the same way with the other test tubes until the absorbance ranges from 0.770 - 0.745. The amount of water determined in this way was the standard dilution for every determination carried out with the starch solution.

3. *Determination in the honey sample solution*

Ten millilitres of honey solution was pipetted into a 50 ml flask and placed in the 40 °C water bath with a second flask containing 10 ml of starch solution. After 15 min, 5 ml of starch solution was pipetted into the honey solution, then it was mixed and the timer was started. At periodic

intervals (for the first 5 min) aliquots of 0.5 ml were removed and it was added 5 ml of diluted iodine solution. Water was added in amounts as determined in the previous point, it was mixed well and then read the absorbance of each separate solution at 660 nm against a water blank in a 1 cm cell.

The intervals after the first removal from the reaction flask had to be timed in a way that 3 to 4 values were obtained in a range of the two absorbances 0.456 and 0.155 (linear range).

4. *Reaction blank*

Ten millilitres of sample solution were added according to the previous point to 5 ml of water and mixed thoroughly. Then, 0.5 ml of the solution were removed and 5 ml of dilute iodine solution was added. Water was added in amounts as determined in *Calibration of the Starch Solution* point, mixed well and then it was read the absorbance of each separate solution at 660 nm against a water blank in a 1 cm cell.

The diastase activity was calculated as diastase number (DN) as follows:

$$DN = 300/ t_x$$

<div align="right">*Equation 7*</div>

t_x = reaction time in minutes obtained as follows:

the absorbance values were plotted against the corresponding reaction times in minutes. The regression line was drawn through the measuring points in the range of A=0.155 to 0.456 in order to determine the time t_x for A=0.235.

- **1. Preparation of the sample solution:**
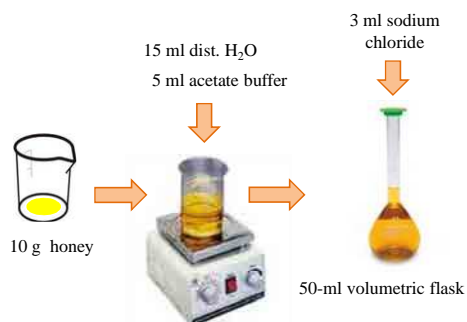
15 ml dist. H$_2$O

5 ml acetate buffer

3 ml sodium chloride

10 g honey

50-ml volumetric flask

- **2. Calibration of the starch solution**:

10 ml water + starch solution

20, 21, 22, 23, 24, 25 ml water

5 ml iodine

5 ml

Read absorbance at 660 nm

0.770-0.745

- **3. Determination in the honey sample solution**

5 ml

15 min

10 ml honey

starch solution

5 ml iodine solution + water

40 °C

5 min

660 nm

- **4. Reaction blank**

10 ml sample solution

5 ml iodine solution + water

660 nm
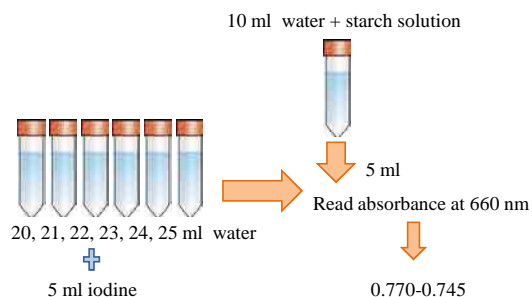
Picture 16. Scheme of the diastase activity determination.

5.2.3. Sugar content determination by HPLC

The sugar (glucose, fructose and sucrose) composition was determined using an HPLC device (Waters HPLC, Ireland) fitted with a refractive index detector (Waters RI 2414) at 25 °C. A honey sample of 1 g was dissolved in 19 ml Milli-Q water, filtered through a 0.22 µm nylon filter into an HPLC vial, capped and injected (20 µl) into the HPLC. The HPLC column was Waters

Carbohydrate Analysis (3.9 x 300 mm). The mobile phase was acetonitrile (HPLC-grade)/water (80/20, v/v) at a flow rate of 1.2 ml/min. External calibration curves constructed from standard solutions of glucose, fructose and sucrose (Panreac, Barcelona, Spain) were used to quantify the amount of sugars in the sample. Results are expressed as gram sugar per 100 g of honey.

5.2.4. Determination of the water activity

Water activity (a_w) of samples was measured at 20 °C using a precision portable measuring device Novasina MS1 set Aw (Novasina, Switzerland). The determination of a_w values was always performed twice; before and after the treatments. Measurements were performed twice.

5.2.5. Honey sensory analysis

The sensory evaluation consisted of a Paired Comparison Preference Test. These types of tests supply information about people's likes and dislikes of a product. They are not intended to evaluate specific characteristics, such as crunchiness or smoothness. Paired preference tests are popular in part because of their simplicity and because the mimic what consumers do when purchasing (choosing among alternatives) (H. T. Lawless and H. Heymann, 2010).

The method requires the subject to indicate which one of the coded products is preferred in order to determine if significant differences exist in preference between these two products. Preference test provides evidence of whether one product is preferred over another.

A frequently used option allows the inclusion of a "no preference" as a third choice, while another option allows the inclusion of a fourth choice, "dislike both equally". However, with small numbers of subjects, permitting ties (no-preference test) reduces the statistical power (i.e. reduces the likehood of finding a difference). In the sensory test situation where the number of the subjects is usually fewer than fifty, the no-preference option is less desirable (H. Stone, J. L.Sidel, 2004) . The more subjects who do not or cannot make the choice of, prefer A or prefer B, the smaller will be the database and the larger the difference in preference needed in order for it to be statistically significant. Moreover, many of the methods for analysis are based on the

binomial theorem and do not permit the inclusion of no-preference ties (H. T. Lawless and H. Heymann, 2010). Replication is not common in preference tests. However recent research has shown that replication will enhance the consumer's discrimination among products in an acceptance test. Also, replication can provide evidence as to whether there are stable segments of consumers who prefer different versions of a product.

The sample preparation was made following M.I.Piana *et al.*(2004) recommendations:

Sample preparation

Sampling beakers

Honey samples were placed in odourless glass beakers, presented in a homogeneous and anonymous way (identical containers with no distinguishing marks apart from the identification code), as Picture 17 shows.

Preparation of sample

Each sample (heat-treated and ultrasound-treated) was given a random three-figure code; about 50 g of the sample were placed in the sampling beaker, which was then covered with s suitable lid (i.e. cling film). The honey was transferred in such a way as to minimize any alteration due to manipulation. The sample temperature was between 18 and 25°C. The evaluation took place within 24 h after the sample treatment and preparation.

General conditions

Evaluators were asked to abstain smoking, eating and drinking anything except water for 30 min before the evaluation. The sessions were timed to take place at least 2 h after the main meals.

Evaluation of the sensory characteristics

Each evaluator was given the material necessary for sensory analysis and evaluation form, which is presented in Picture 18.

Picture 17. Top: Honey taste presentation. Bottom: Evaluators tasting honey.

HONEY

INSTRUCTIONS

You will be presented with two samples of honey. Then, you will be asked to choose and mark the sample you like the most.

A. VISUAL CHARACTERISTICS

1. Raise and observe the beaker to your left. Proceed likewise with the one to your right.
   Now that you've observed both products, which one do you prefer in terms of **appearance**? Please choose one:

   ☐   ☐

   <u>463</u>   <u>189</u>

2. Please   comment   on   the   reasons   for   your   choice:
   _____
   _____
   _____
   _____

B. OLFACTORY CHARACTERISTICS

1. Take the beaker on your left. Move the honey around with the plastic spoon or just swirl it. Breathe in for a few seconds. Wait for approx. 10 seconds and proceed likewise with the beaker to your right.
   Now that you've smelled both products, which one do you prefer in terms of **aroma**? Please choose one:

   ☐   ☐

   <u>463</u>   <u>189</u>

2. Please   comment   on   the   reasons   for   your   choice:
   _____
   _____
   _____
   _____

C. TEXTURAL CHARACTERISTICS

1. Please taste the honey to your left and <u>concentrate on its mouth-feel</u>. Wait for approx. 1 minute. Proceed likewise with the honey to your right.
   Now that you've tasted both products, which one do you prefer in terms of **texture**? Please choose one:

   ☐   ☐

   <u>463</u>   <u>189</u>

2. Please   comment   on   the   reasons   for   your   choice:
   _____
   _____
   _____
   _____

D. FLAVOUR CHARACTERISTICS

1. Taste the honey on your left and <u>concentrate on its flavour this time</u>. Wait for approx. 1 minute. Proceed likewise with the honey to your right,
Now that you've tasted both products, which one do you prefer in terms of **flavour**? Please choose one:

<u>463</u>   <u>189</u>

2. Please   comment   on   the   reasons   for   your   choice:
_____
_____
_____
_____

E. OVERALL

1. Which one do you prefer overall?

<u>463</u>   <u>189</u>

2. Please   comment   on   the   reasons   for   your   choice:
_____
_____
_____
_____

Picture 18. Evaluation form.

5.2.6. Statistical analysis

Minitab 15 was used to perform statistical analyses of the data obtained. Principal Component Analysis (PCA) and ANOVA were used to study the effect of ultrasound and heating at different time and temperatures on HMF, diastase activity and sugar content. F-test (α=0.05) was used to examine for any significant differences among honey treatments.

5.3. Results and Discussion

5.3.1. Determination of Hidroxymethylfurfural (HMF) content in honey by HPLC

Hidroxymethylfurfural (HMF) is produced by acid catalyzed dehydration of hexoses. In honey, glucose and fructose decompose in the presence of gluconic acid to form HMF. There is virtually no HMF in fresh honey, and its formation is influenced by several factors such as temperature and time of heating, storage conditions, pH and floral source from which the honey has been extracted (B. Fallico *et al.*, 2004). The presence of organic acids and low water activity also favours the production of HMF (S.Ajlouni and P. Sujirapinyokul, 2009). Honey is heated differently worldwide at various stages of its processing to reduce viscosity, dissolve crystals and destroy yeast. However, such heat treatments increase the HMF content of honey. Several authors reported that honey is treated at temperatures of 71-72 °C for 30 min (R. A. Grout, 1949). Other authors related the honey to be treated at 63°C for 30 min or 77 °C for few seconds (flash pasteurization) (E. Crane, 1975), 55 °C for 16 h or 72 °C for 2 min (B. d'Arcy, 2007) or 78 °C for 6–7 min (E. Tosi *et al.*, 2004) . Dimins *et al.* (2006) point out the formation of HMF at 60 °C; however, its formation is due to a combination of temperature and time. For example, heating the honey at 90 °C for 15 min results in a concentration of 0.80 ± 0.25 mg HMF/kg, while a concentration of 1.33 ± 0.11 mg/kg is found at 75 °C for 90 min (I. Turhan *et al.*, 2008). S.Ajlouni and P. Sujirapinyokul (2009) found an increase of 8 mg/kg of HMF heating the honey at 85 °C for just 2 min. Although in the last years many works have focused in the kinetics formation of HMF in honey (E. Tosi *et al.*, 2002; 2004; I. Turhan *et al.*, 2007; B. Fallico *et al.*, 2004), this compound is formed differently depending on the heat regime applied and the type (composition) of honey. Therefore, HMF content can be used as an indicator to detect the heat damage and adulteration of honey.

In a first approximation, rosemary commercially honey was used (Bona Mel Organic, S.L. Organic Production, CE control System, Alicante) for the preliminary tests. Honey samples were treated by ultrasounds varying the parameters of temperature (40, 50 and 60 °C), time of

treatment (20, 40 and 60 min) and power output (50, 100 and 200 W) in order to see whether there was a relationship between these parameters and which ones were the optimums.

Table 12 shows the different treatments applied and the HMF formation. The initial value, that is honey before being treated, ranged between 10.36 and 11.86 mg HMF/kg of honey. E. Tosi *et al.* (2004) reported in fresh honeys 10 mg/kg HMF levels are found. However, C. Pérez-Arquillué *et al.* (1995) and I. Escriche *et al.* (2009) analyzed fresh rosemary honey and obtained values of 3.0-3.7 and 3.1 -3.8 mg HMF/kg of honey, respectively. So these higher values could be probably due to a heat treatment suffered by the honey when processed or because this honey is not completely fresh (> 5 months). Nevertheless, the objective was to quantify the increase of HMF in honey when treated by ultrasounds.

From the results obtained (see Table 12) it can be inferred that after different combinations of treatments there was no significant ($P > 0.05$) HMF increase (and it was still lower than 40 mg kg^{-1}) in accordance with international limits. However, having 3 parameters (and thus too much combinations) it cannot be deduced if it does exist a relationship between the temperature-time-power in the HFM formation.

Table 12. HMF formation in honey after being treated (mg kg-1).

Sample number	US Power (W)	Temperature (°C)	US treatment time (min)	HMF (mg kg^{-1})
control	-	-	-	10.35
control	-	-	-	11.86
1	50	40	40	11.10
2	200	40	40	10.64
3	50	60	40	14.24
4	200	60	40	13.11
5	50	50	20	11.54
6	200	50	20	12.32
7	50	50	60	11.85
8	200	50	60	12.48
9	100	40	20	10.35
10	100	60	20	11.43
11	100	40	60	10.60
12	100	60	60	15.93
13	100	50	40	11.96
14	100	50	50	11.23
15	100	60	40	11.70

Thus, a second experiment was carried out fixing the ultrasound bath power (200 W) and varying the temperature and treatment time.

In this second experiment, honey samples were treated at 40 °C, 50 °C and 60 °C during 20, 40 and 60 minutes through an ultrasound bath (US) and the HMF content was analyzed. These parameters were chosen taking into account the results from chapter one, that is, the rheological behaviour, so it could be possible to establish the best conditions for liquefying the honey without compromising its quality.

The HMF content of the heat-treated (HT) samples was also determined in order to verify whether an US treatment increases the HMF values or if this compound is not influenced by the US waves.

From the results obtained in Table 13, it can be noticed that heating at 40 °C, 50 °C or 60 °C for 60 min in rosemary honey type, the HMF content was still far away from the international limits (lower than 40 mg kg^{-1}).

These results are in accordance with I. Turhan *et al.* (2008) which also did not found a significant HMF increase ($P > 0.05$) after heating the samples at 90 °C for up to 90 min in floral honeys, and up to 75 min in honeydew honeys. It was also noticed that the US-treated samples and the HT samples present almost the same HMF content after applying their respective treatments. The ANOVA test corroborated no significant differences between US and HT samples ($P > 0.05$), indicating that the US waves did not influenced the HMF formation.

The activation energy (E_a) is defined as the minimum energy required starting a process. The Arrhenius equation gives the quantitative basis of the relationship between the E_a and the rate at which a reaction proceeds.

HMF in honey was fitted to a first-order reaction (Eq. (5)), and activation energies (E_a) were calculated according to Arrhenius model (Eq. (6)).

The result of E_a value calculated was 31.06 kcal/mol, which is very similar to those values found by other authors. B. Fallico *et al.* (2004) studied the HMF formation in floral honeys during thermal process at 50 °C, 70 °C and 100 °C up to 60 h obtaining E_a values of 32.50 and 43.60 kcal/mol. Moreover, E. Tosi *et al.* (2002) studied the kinetic formation of HMF during transient and isothermal heating stages in a time range from 15 to 60 seconds at a range temperature from 80 °C to 140 °C and their E_a value was 54.00 kcal/mol I. Turhan *et al.* (2008). also calculated the E_a value for the formation rate of HMF at 50 °C, 70 °C and 90 °C in time range from 15 to 90 min. They obtained an E_a value of 22.32 kcal/mol. The results mentioned above demonstrate that the US treatment does not overcome the energy barrier to carry out the reaction of HMF formation.

Table 13 HMF formation of different honeys at different temperatures (mg·kg^{-1}) at a power level of 200 W.

		US-treated	Heat Treated
40 °C 20min	min	11.4	10.92
	max	14.27	14.24
	average	**12.93**	**12.12**
40 °C 40 min	min	9.67	11.21
	max	15.92	13.54
	average	**12.96**	**12.4**
40°C 60min	min	9.34	11.72
	max	14.17	12.77
	average	**13.00**	**11.07**
50 °C 20min	min	10.26	11.1
	max	15.00	11.14
	average	**13.00**	**11.12**
50°C 40min	min	9.93	11.14
	max	16.00	11.4
	average	**13.07**	**11.12**
50 °C 60min	min	9.77	11.14
	max	12.57	12.16
	average	**13.4**	**11.53**
60 °C 20min	min	9.51	11.77
	max	13.65	15.91
	average	**12.96**	**13.63**
60 °C 40min	min	9.8	11.18
	max	14.14	14.11
	average	**13.09**	**13.28**
60 °C 60min	min	9.66	11.21
	max	14.65	15.00
	average	**13.25**	**12.09**

Principal component analysis (PCA) is an appropriate tool for analyzing data when it has been obtained measures on a number of observed variables (i.e. temperature, time, power) and it and it is believed there is some redundancy in those variables (I. T. Joylliffe, 2002). In this case, redundancy means that some of the variables are correlated with one another, possibly because they are measuring the same construct (HMF). Because of this redundancy, it is believed it should be possible to reduce the observed variables into a smaller number of principal components that will account for most of the variance in the observed variables. PCA is mathematically defined as an orthogonal linear transformation that converts the data to a new coordinate system (I. T. Joylliffe, 2002).   This transformation is defined in such a way that the greatest variance (eigenvalue) by any projection of the data comes to lie on the first coordinate (called the first principal component), the second greatest variance on the second coordinate, and so on. The number of principal components is less than or equal to the number of original variables.

The standardized PCA (Figure 10) revealed a relationship between Temperature and the HMF formation. As mentioned before, the eigenvector with the highest eigenvalue is the principle component of the data set. From Figure 10 and Table 14, the first principal component has a variance (eigenvalue) of 1.7681, which in turn explains the 44.2% of the total variance.

Coefficients below PC1 show how to calculate principal component:

PC1=-0.012*Power + 0.650*Temp. + 0.277*Time + 0.708*HMF

The second principal component has a variance of 1.0037 and explains the 25.1% of the variability of the data. It is calculated from the original data using the coefficients that appear under PC2. Together, the two and three first principal components represent 69.3% and 94.3% respectively, of the total variability.

Thus, from the explanation above, the X axis describes the linear relationship between the variable of Temperature and the formation of HMF (Figure 10) and accounts the 44.2% of the total variance. The Y axis describes the linear relationship between the variable of Time, Temperature and Power and represents 25% of the variability of the data. The angle of the variable Power respect the formation of HMF is orthogonal, which means that the concentration of HMF will not be influenced respective of whether Power is increased or not. The Temperature change is a factor that influences the formation of HMF because it is more closely linked to the

formation of HMF. The higher the Temperature the higher the HMF concentration. These two variables increase together. The variable of Time also influences the HMF formation but not as much as the variable of Temperature nor as little as the variable of Power.

For example, sample 9 is on the left part of scheme (Figure 10). Medium Power (level 2), low Temperature (40 °C) and low treatment Time (20 min) was applied to this sample, which lead to a very low content of HMF, among the samples. While sample 3 which is on the right side of the scheme had low Power (level 1), high Temperature (60 °C) and medium treatment (20 min) Time had high concentration of HMF. It was followed the same proceeding for the analysis of the rest of the samples.
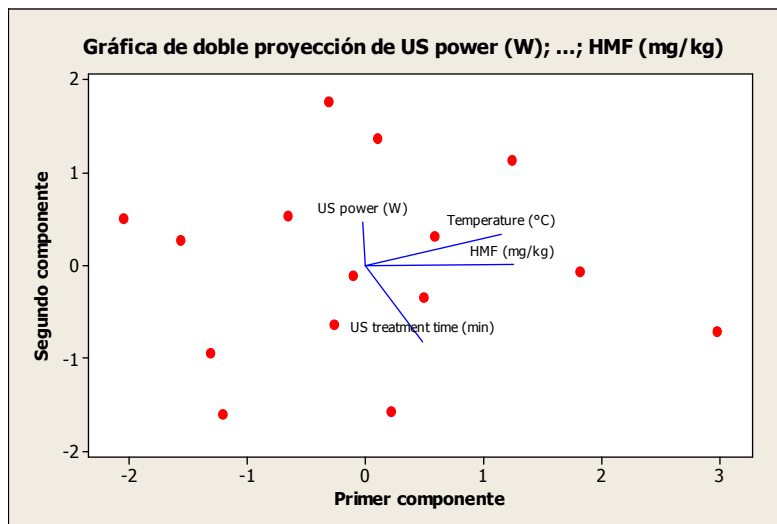
Figure 10. Hidroxymethylfurfural Principal Component Analysis. The directions represent the Principal Components (PC) associated with the sample HMF.

Table 14. Analysis of HMF values and eigenvectors of the correlation matrix

Eigenvalue	1.7681	1.0037	0.9997	0.2285
Proportion	0.442	0.251	0.25	0.057
Cumulative	0.442	0.693	0.943	1

Variable	PC1	PC2	PC3	PC4
Power	-0.012	0.474	0.88	0.03
Temperature	0.65	0.342	-0.198	0.649
Time	0.277	-0.811	0.431	0.282
HMF	0.708	0.011	0.028	-0.706

5.3.2. Evaluation of diastase activity (DN)

The diastase (amylase) activity is usually expressed as diastase number (DN) and also is known as Gothe units. A Gothe unit is defined as ml of 1% starch solution hydrolyzed at 40 °C for one hour by the enzyme present in 1 g of honey (International Honey Commission, 2002).

According to the Honey Quality and International Regulatory Standards, from the International Honey Commission, and the Official Spanish State Bulletin (BOE 1049/2003) the diastase activity must not be less than or equal to 8, expressed as diastase number (DN). Diastase activity should be determined after processing and blending or for all retail honey. The Codex Alimentarius (1998) has established the minimum diastase activity value of 3 DN, for honeys with natural low enzyme content. In honeys with a DN less than 8 and higher than or equal to 3 DN, then the hidroxymethylfurfural (HMF) must not be higher than 15 mg/kg. If DN is equal to or higher than 8, then the HMF limit is 60 mg/kg.

In the present research, the initial amylase activity (control sample) ranged from 9.35 to 10.34 (Table 15) in the tested commercial honey.

Table 15. Diastase activity, expressed as diastase number (DN) of control and 15 US-treated samples.

Sample number	US Power (W)	Temperature (°C)	US treatment time (min)	DN
control	-	-	-	10.35
control	-	-	-	9.38
1	50	40	40	8.90
2	200	40	40	10.64
3	50	60	40	10.24
4	200	60	40	8.11
5	50	50	20	11.54
6	200	50	20	9.32
7	50	50	60	8.85
8	200	50	60	7.48
9	100	40	20	9.35
10	100	60	20	9.43
11	100	40	60	8.60
12	100	60	60	9.93
13	100	50	40	8.96
14	100	50	50	9.23
15	100	60	40	8.70

Although these values are relatively low compared with other honey types (e.g. Heather (*Calluna, Erica sp.*) DN of 22) , they accord with other rosemary honey values of 3-10 DN E. Mendes *et al.*, 1998; L.P. Oddo et al, 2006).

L.P. Oddo *et al.* (2006) suggest that these lower values may be because the rosemary honey type is collected in early spring when the colony feeds brood and young bees, whose glands produce fewer enzymes. Even thought, heating honey samples in a water bath for 20, 40 and 60 minutes at 40 °C, 50 °C and 60 °C did not affect the level of amylase inactivation (Table 16). As for the US treatments, no one of the different US-treated samples presented a lower DN value. The ANOVA

test also corroborated there were no significant differences ($P > 0.05$). So, applying a 40 kHz frequency for the times mentioned above does not seem to affect the enzymatic activity as it can be show in Table 17.

A study of S.Ajlouni and P. Sujirapinyokul (2009) showed that the honey samples with a low DN values and the honey samples with a high initial DN values, lost similar proportions (19.0–19.5 %) of their amylase activities when heated for 2 min at 85 °C. The same data also showed that all tested honey samples, could be heated at 85 °C, and the remaining DN was still above the minimum limit (8 DN). Consequently, it has been recommended that other quality indicators, such as invertase activity, which is more heat-sensitive than amylase, should be used (L.P. Oddo et al, 2006; F. Dimins *et al.*, 2006). Moreover, a study by E. Tosi *et al.*(2008) showed that destroying all amylase activity required heating honey at 80 °C for 1.2 h, while it only took only 8.6 min for the inactivation of invertase present.

Table 16. Diastase activity, expressed as diastase number (DN) of heat-treated samples.

Sample	DN
40 °C 20 min	8.52
40 °C 40 min	9.09
50 °C 20 min	9.32
50 °C 40 min	9.11
60 °C 20 min	7.89
60 °C 40 min	8.33
60 °C 60 min	9.38

Table 17. Maximum, minimum, mean and standard deviation of Diastase activity, expressed as diastase number (DN) of US-treated samples, HT samples and control samples at all temperature range.

DN US-treated samples	
Max	11.54
Min	7.48
Mean	**9.28**
SD	**1.01**
DN Heat treated samples	
Max	9.38
Min	6.25
Mean	**8.64**
SD	**0.59**
DN Control	
Mean	**9.86**
SD	**0.69**

5.3.3. Sugar content determination by HPLC

Sugars contained in nectars are mainly fructose, glucose and sucrose, but their relative proportions are usually rather variable; however, they are quite consistent for certain botanical families (R. Mateo and F. Bosch-Reig, 1996).

In respect to reducing sugars (fructose and glucose), EC Directive 2001/110 imposes reducing sugars ≥ 60 g/100 g, except for honeydew honey, which is ≥ 45 g/100 g. Non-reducing sugars (apparent sucrose) are set to be ≥ 5 g/100 g for the majority of honeys, except for citrus and eucalyptus honeys, which have higher limits (≥ 10 g/100 g), as well as lavender honeys (≥ 15 g/100 g) (EC Directive 2001/110).

The initial sugar content, in terms of the fructose and glucose mean values agreed with results reported by C. Pérez-Arquillué *et al.* (1995) and R. Mateo and F. Bosch-Reig (1996) of 36 % fructose and 31 % glucose, and no significant ($P > 0.05$) variation of the these monosaccharides was found in the HT (Table 19) or the US-treated samples after applying their respective

treatments (Table 18). However, other authors such as K. Zurcher *et al.* (1974) and M. Gonnet (1979) have obtained higher values of these monosaccharides in contrast to lower values obtained by M.A. Mohamed *et al.* (1982) of 32.90 % for fructose and 24.95 % for glucose for the rosemary honey type (Tables 18 and 19).

The ratio fructose/glucose obtained in this research was 1.14 ± 0.01, a proportion which is almost in the range proposed by J. Pourtallier, J and Y. Taliercio (1970) for the rosemary honey (1.06-1.13). C. Pérez-Arquillué *et al.* (1995) also obtained a ratio which is as well slightly out of the range (1.17 ± 0.01) for the rosemary honey type.

Table 18. Concentration of glucose, fructose and sucrose in the control and the US treated honey

Sample number	US Power (w)	Temperature (°C)	US Treatment time (min)	Fructose (f)	Glucose (g)	Sucrose	f/g	f+g
Control	-	-	-	36.03	31.69	0.71	1.13	67.72
1	50	40	40	35.31	31.01	0.60	1.13	66.32
2	200	40	40	35.22	31.85	0.67	1.10	67.07
3	50	60	40	35.77	31.02	0.61	1.15	66.79
4	200	60	40	36.12	31.44	0.70	1.14	67.56
5	50	50	20	35.66	30.97	0.68	1.15	66.63
6	200	50	20	35.78	31.89	0.72	1.12	67.67
7	50	50	60	36.06	31.02	0.69	1.16	67.08
8	200	50	60	35.07	31.04	0.80	1.12	66.11
9	100	40	20	35.88	30.92	0.78	1.16	66.80
10	100	60	20	35.70	31.54	0.72	1.13	67.24
11	100	40	60	35.13	30.79	0.80	1.14	65.92
12	100	60	60	35.72	30.97	0.80	1.15	66.69
13	100	50	40	35.69	30.84	0.71	1.15	66.53
14	100	50	50	35.62	30.99	0.73	1.14	66.61
15	100	60	40	35.71	30.77	0.70	1.16	66.48
			average	**35.63**	**31.14**	**0.71**	**1.14**	**66.77**
			SD	**0.31**	**0.35**	**0.06**	**0.02**	**-**

samples (g/100 g) and fructose/glucose ratios.

The ratio fructose/glucose is important for determining the tendency of honey to crystallize. High values have been associated with liquid or slow-granulating honey. What crystallizes is the glucose, due to its lower solubility. Fructose is more soluble in water than glucose and will remain fluid (K. Hamdan, 2012). When glucose crystallizes, it separates from water and takes the form of tiny crystals. As the crystallization progresses and more glucose crystallizes, those

crystals spread throughout the honey. The solution changes to a stable saturated form, and ultimately the honey becomes thick or crystallized. J. White *et al.* (1962) presented a useful table of sugar composition which they related to honey granulating tendency. They attributed a value of 1.35 to completely liquid honey, 1.25 to few scattered crystals, 1.18 to few clumps of crystals, 1.16 -1.11 to half granulated and 1.10 to completely granulated honey. Hence, the honey analyzed in this research can be classified as a honey with an intermediate tendency of granulation. Other authors have also classified the rosemary honey type in medium-crystallization rate (E.Crane 1975; K. Hamdan, 2012).

The content of sucrose in this work was found to be 0.71 ± 0.06, this value is similar to other authors (M.A. Mohamed *et al.*,1982; C. Pérez-Arquillué *et al.*,1995)   and no alteration of the sugar content was found after applying the temperature or any of the US treatments.

Table 19. Concentration of glucose, fructose and sucrose in the HT honey samples (g/100 g) and fructose/glucose ratios.

Sample	Fructose (f)	Glucose (g)	Sucrose	f/g	f+g
40 ^{0}C 60 min	35.90	31.07	0.72	1.16	66.97
50 ^{0}C 60 min	36.08	31.01	0.69	1.16	67.09
60 ^{0}C 60 min	35.89	31.46	0.79	1.14	67.35

5.3.4. Determination of the water activity

The water activity (a_w) represents the ratio of the water vapor pressure of the food (p) to the water vapor pressure of pure water (po) under the same conditions and it is expressed as a fraction (a_w = p/po) . It is a measure of the water available for bacterial growth.

The water activity needed for development of microorganisms is below 0.98 and depends on the class of microorganisms; around 0.70 for mould; 0.80 for yeast and 0.90 for bacteria. Osmophilic yeast are specialists which have an obligate need for high sugar concentrations and are able to grow to a minimal water activity until 0.6. Such osmophilic yeast are causing honey fermentation

(R.A. Gleiter *et al.*, 2006). Moreover, a_w has great importance during the extraction and storage of honey, as it influence the texture, stability and shelf life of honey. R.A. Gleiter *et al.* (2006) and S. Gomes *et al.* (2010) analysed the connection between water activity and honey crystallisation. They found the water activities of crystallised honeys were higher than those of liquid honeys having the same water content. However no differences among the water activities of different types of honeys could be found in crystallised state.

Honey's water activity varies between 0.5 (16% moisture) and 0.6 (18.3% moisture) in the 4-37 °C temperature range, and it is therefore shelf stable for a reasonable period of time. The low a_w (high osmotic environment) does not support microbial growth, preventing fermentation of honey by osmophilic yeast (A. Lazaridou *et al.,* 2004).

The intial a_w value of the rosemary honey obtained was 0.613-0.614. Moreover, the a_w of the US-treated samples varied within the range 0.587–0.589 indicating that an US treatment could slightly decrease the a_w value. Water activity although not being legislated, meet the values reported by other authors for honeys (A. Terrab *et al.*, 2002; A. Lazaridou *et al.,* 2004; S. Gomes *et al.*, 2010).

5.3.5. Honey sensory analysis

The sensory evaluation consisted of a Paired Comparison Preference Test. The analysis was based on the evaluation of visual, textural and olfactory-gustatory characteristics of ultrasound-treated (US) and heat-treated (HT) honey by a consumer panel. The subjects used in this test (10 persons) were frequent users of honey. The evaluators were asked to indicate which one of the coded honey they preferred the most, between the US and the HT samples.

Although sensory analysis is not one of the objectives of this thesis, it was thought appropriate to organize a small taste to strengthen and complement the analysis, and to have a general idea or approximation of the perceptions in the final consumer.

The objective of the taste was to determine whether significant differences exist in preference between these two products. In other words, to verify whether an ultrasound treatment affects to the sensorial properties of honey.

Since the subjects involved were fewer than fifty, the non-preference choice was no contemplated, in order to be the results statistically significant.

Replication is not common in preference tests. However recent research has shown that replication will enhance the consumer's discrimination among products in an acceptance test (H. T. Lawless and H. Heymann, 2010). Also, replication can provide evidence as to whether there are stable segments of consumers who prefer different versions of a product tests (S. Kemp *et al.*, 2009). Hence, the taste was repeated by the same consumers a week after the first taste.

The rather striking fact was the wide variety of the consumers' response. Some of the comments described in the form are stated below:

463 is the ultrasonicated sample and 189 belong to the heat-treated sample.

Visual evaluation:

"*Sample 463 seems to have a nicer appearance, look less thick in texture and have a less cloud appearance*".

"*Sample 463 has less bubble, more homogeneous, a bit clearer*".

"*Sample 463 looks clearer, more "fresh", sample 189 looks very thick*".

"189, I prefer viscous honey".

Olfactory characteristics:

"I believe the aroma is more intense (463 sample)".

"Sample 189 seemed to have a stronger smell".

"Can't find difference".

"The 189 sample is more smooth and good aroma".

"*Sample 463 has a stronger smell*".

Textural characteristics:

"Sample 463 is less thick, less viscous; I liked it more than the other".

"Sample 189 feels a lot thicker, like traditional honey, I preferred it".

"More liquid, fluid (sample 463) I liked more".

"The 189 is more viscous than the other one, I preferred over 463".

"I like more 463, it's smoother, more "liquid".

"No real preference".

"189 is thicker, I liked more".

Flavour characteristics:
"Flavours are both good".
"Can't find any difference".
"I prefer 463; it seems to me less sweet but more complex".
"Don't like aftertaste of 463".
"I don't know why I prefer 189".
"Very similar but I prefer 189".
"463 tastes sweeter and has a stronger flavour".

In spite of that non-preference choice was not presented as an option, many consumers were still likely to use it as a mean as of avoiding "giving the wrong answer" or, an easier option than making a choice, despite the fact they probably did have a preference.

Moreover, it was observed (more than 5 cases) that consumers changed up their minds, since their preference patterns varied in the second taste. Table 20 presents the results of the first and second taste.

Therefore, due to the wide variety of preferences among consumers, no significant preference was observed in any of the attributes. Significance in two tailed test is found by simple lookup Table 21 (H. T. Lawless and H. Heymann, 2010).

The only exception observed was the visual evaluation. Adding the results from both tastes, it was found in Table 21 that the value for 20 panellists at alpha 5% was 15. The obtained value of 17 is greater than this minimum and therefore the consumers had a significant preference for US sample over the HT sample. Oppositely, as none of the other attributes scored 15 points, no significance preference was observed.

Table 20. Honey taste, Paired Comparison Preference Test results

	first taste		
	HT	US	no-preference
visual	2	8	0
olfactory	3	4	3
textural	4	5	1
flavour	5	5	0
	second taste		
	HT	US	no-preference
visual	0	9	1
olfactory	3	3	4
textural	5	5	0
flavour	4	4	2

Table 21. Minimum value (X) required for a significant preference.

N	X	N	X	N	X	N	X
20	15	41	28	62	40	83	51
21	16	42	28	63	40	84	52
22	17	43	29	64	41	85	53
23	17	44	29	65	41	86	53
24	18	45	30	66	42	87	54
25	18	46	31	67	43	88	54
26	19	47	31	68	43	89	55
27	20	48	32	69	44	90	55
28	20	49	32	70	44	91	56
29	21	50	33	71	45	92	56
30	21	51	34	72	46	93	57
31	22	52	34	73	46	94	57
32	23	53	35	74	47	95	58
33	23	54	35	75	48	96	59
34	24	55	36	76	48	97	59
35	24	56	36	77	49	98	60
36	25	57	37	78	49	99	60
37	25	58	37	79	50	100	61
38	26	59	38	80	50	105	64
39	27	60	39	81	50	110	66
40	27	61	39	82	51	115	69

Note: N is the number of consumers, X is the minimum required in the larger of the two segments.

Choice tests indicate the direction of preferences for the product but the results give no indication of the size of the preference. Hence, it cannot be concluded whether consumers preferred the sonicated honey over the only heat-treated one, but at least it can be concluded they did not rejected.

5.4. Conclusions

Bearing in mind the test conditions of the first aim (refer to Chapter 1) the present Chapter analyzed the main honey quality parameters when exposed to a treatment process for liquefying crystallized honey using high-power low-frequency ultrasounds.

1. Different combinations of ultrasound treatment did not increase significantly the hydroxymethylfurfural (HMF) content of honey. Moreover, HMF in honey was fitted to a first-order reaction and activation energies (E_a) were calculated according to Arrhenius model. The E_a obtained revealed that an US treatment did not overcome the energy barrier to carry out the reaction of HMF formation.

2. The diastase activity was either influenced by the ultrasound treatment as no significant decrease was observed.

3. The sugar content in terms of fructose (36%) and glucose (31%) was not altered after sonication, and all ratios and sugar proportions remained constant after sonication. The fructose/glucose ratio of 1.14 ± 0.01 let associating the honey with an intermediate tendency of granulation.

4. The initial a_w value of the rosemary honey obtained was 0.613. Moreover, the a_w of the US-treated samples varied within the range 0.587–0.589 indicating that an US treatment could slightly decrease the a_w value and thus preventing the deterioration of honey by microbial contamination.

5. The sensory analysis gave an idea of the consumer liking, who revealed no preference between the US and HT samples. The only exception observed was the visual evaluation, where there was significant preference since 17 of 20 preferred the US sample, while 2 consumers preferred the HT and 1 consumer wrote down "no-preference". However, these results are only an approximation of consumer's preferences.

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6. CHAPTER 3: ULTRASOUNDS ON HONEY DECONTAMINATION AND ANTIMICROBIAL ACTIVITY
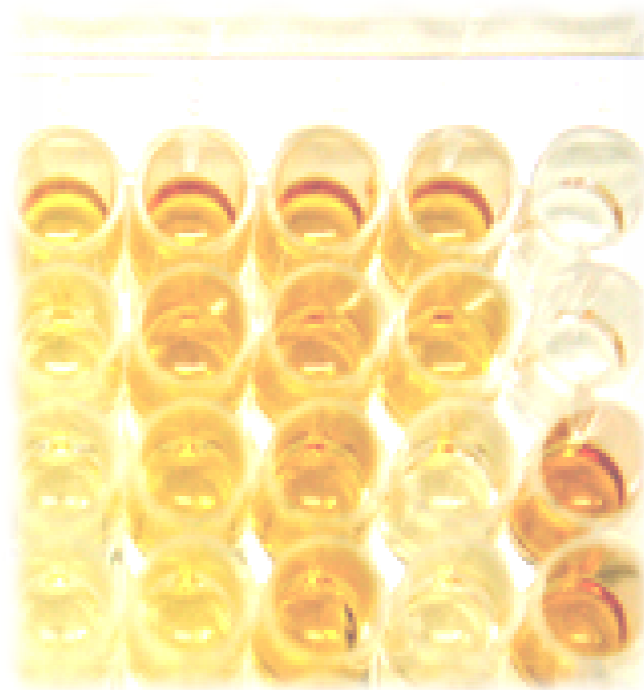
6.1. Introduction

Honey is a very concentrated sugar solution (60-80 %) with a high osmotic pressure (water activity < 0.6). The combination of these factors suppresses the growth of spoilage bacteria and contributes to the stability of the product without the necessity of particular storage conditions (S. Bogdanov, 2002; C.Voidarou *et al.*, 2011). However, **the presence of a small number of bacteria species is anticipated in unpasteurized honeys**, comprising their natural microflora (J. Snowdown and D. O.Cliver, 1996). On the other hand, variable amounts of hydrogen peroxide, free radical production, increased osmolarity, acidity, water activity, volatiles, organic acids, phenolic compounds and beeswax, among others, are proposed by many researches as the contributing factors for its **antimicrobial activity** (C. Voidarou *et al.*, 2011; P.C. Molan, 1992). **The microbes that may be found in honey are primarily yeasts and spore-forming bacteria**, whereas no vegetative forms of disease-causing bacterial spores have been found in honey (J. White *et al.*, 1963; NHB, 2012). Most research on primary sources of microbes in honey are done to understand the microbial ecology of the honey bee (J. Snowdown and D. O.Cliver, 1996). It appears that pollen is the proximate source of microbes that 'seed' the intestines of bees.

Primary sources of microbial contamination are likely to include pollen, the digestive tracts of honey bees, dust, air, earth, and nectar. Soil and flowers may be also sources of yeasts in honey. These sources are very difficult to control. Secondary sources are those arising from honey manipulation by people that include food handlers, cross-contamination, equipment and buildings (M.S. Finola *et al.*, 2007; NHB, 2012). Because bacteria do not replicate in honey, if high numbers of vegetative bacteria were to be detected, it may indicate contamination from a secondary source (NHB, 2012).

No special techniques for the sampling or analysis of honey, for microbes of significance to human health or honey quality, have been developed and standardized (i.e. neither the Council Directive 2001/110/EC relating to honey, the Official Spanish State Bulletin (BOE 1986/15960) nor the International Honey Commission in its International Harmonised Methods report, provide any data about the maximum permissible microbiological level of honey, and neither the methodology to follow). However, the Codex Standard for Honey (Codex Stan 12-1981)

recommend the honey to be prepared and handled in accordance with the appropriate sections of the Recommended International Code of Practice - General Principles of Food Hygiene recommended by the Codex Alimentarius Commission (CAC/RCP 1-1969), and other relevant Codex texts such as Codes of Hygienic Practice and Codes of Practice. On the other hand, the Codex Standard for Honey sets that honey should comply with the microbiological criteria established in accordance with the Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21-1997)**. It is generally accepted a total bacteria count of 1000 CFU/g of honey and for fungi and yeast less than 100 CFU/g.**

Although the routine microbiological examination of honey might include several different assays, most references in the scientific literature for the detection of microbes in honey are primarily done by a standard plate count, which provides general information of honey microflora (J. Snowdown and D. O.Cliver, 1996;   M.S. Finola *et al.*, 2007; S. Gomes *et al.,* 2009). Additional tests may be needed if unusually high counts are found in honey (J. Snowdown and D. O.Cliver, 1996).

When analyzing and studying the therapeutic properties of honeys, modern science has made it possible to specify their medical significance as bactericidal, bacteriostatic, antiviral, antioxidant, anti-inflammatory, and antitumoral (P.C. Molan, 2001; P.E Lusby *et al.*, 2005; J. Bardy *et al.*, 2008; L. Estevinho *et al.*, 2008). **Honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, Gram-positives, and Gram-negatives** (D.P Mohapatra *et al.*, 2010). Honey has a **potent antibacterial activity** and is very effective in clearing infection in wounds and protecting them from becoming infected (P.C. Molan, 2001; D.P. Mohapatra *et al.*, 2010). It maintains a moist wound environment that promotes healing, and its high viscosity helps to provide a protective barrier to prevent infection. Low concentrations of this known antiseptic are effective against infectious bacteria and can play a role in the wound healing mechanism (P.C. Molan, 1992). In addition, the mild acidity and low level hydrogen peroxide release assists both tissue repair and contributes to the antibacterial activity (A. I. Schepartz and M. H. Subers, 1964). In general, all types of honey have high sugar content but a low water content and acidity, which prevent microbial growth. Most types of **honey generate hydrogen peroxide when diluted** because of the activation of the enzyme

glucose oxidase, which oxidizes glucose to gluconic acid and hydrogen peroxide (A. I. Schepartz and M. H. Subers, 1964; D.P Mohapatra *et al.* 2010). **Hydrogen peroxide is the major contributor to the antimicrobial activity of honey**, and the different concentrations of this compound in different honeys result in their varying antimicrobial effects (P.C. Molan 1992).

Ultrasound has been applied in microbiology for sonication and inactivation of bacteria in food processing (P. Piyasena *et al.*, 2003). The **inactivation of microorganisms by ultrasonic waves has been attributed to the cavitation phenomenon**. Microbubbles of gas and/or vapour formed within a liquid during the rarefaction cycle of the acoustic wave undergo violent collapse during the compression cycle of the wave. This implosion or cavitation releases large amounts of energy, generating temperatures of 5000 K and shock waves with pressures of several atmospheres (T. J. Mason, 1990). Sporulated microorganisms appear to be more resistant to ultrasound than vegetative ones and fungi are more resistant in general than vegetative bacteria (H. Alliger, 1975). The use of ultrasonic waves as a unique preservation factor, however, is unable to effectively kill all the microorganisms since the high levels needed could adversely modify nutritional and sensory properties of the food. This limitation has suggested that ultrasound could be more effective when used in combination with other techniques like heat (thermo-ultrasonication) and pressure (manosonication) for inactivation of some pathogenic and spoilage microorganisms. Microbial inactivation by ultrasound depends on many factors that are critical to the outcome of the process. These factors are design parameters (i.e. ultrasonic power and wave amplitude, temperature, volume of the food to be processed), product parameters (i.e. composition and physical properties of the food) and microbial characteristics, as well as environmental stress factors used in combination.

Therefore, the purpose of the present Chapter is to evaluate the effect of different combinations of ultrasound treatment (power, temperature and duration) on honey **microbiological quality**, and on the other hand, to assess whether the **biological activity** of honey is affected after an ultrasound treatment. The main objectives are:

- Evaluate the degree of **microbiological decontamination** of honey after an ultrasound treatment.

- Analyze the *in vitro* **antimicrobial and antifungal activities** of ultrasonicated honey against several types of microorganisms; *Candida amapae*, *Saccharomyces cerevisiae*, *Escherichia coli,* and *Bacillus cereus.*

6.2. Material and methods

6.2.1. Honey samples

Crystallized rosemary *(Rosmarinus officinalis)* honey was obtained from a local producer (Viadiu Ltd., Caldes de Montbuí Spain). The samples were delivered to the laboratory premises within 24 h after collection, homogenized in a glass bottle (1 l) and stored at room temperature for the study. Samples were then treated (refer to Material and Methods Chapter 1).

6.2.2. Microbiological analysis

Aliquots of 1 gram of honey samples (ultrasound-treated, heat-treated and raw honey) were placed in sterile Petri dishes and incubated in Nutrient Agar (NA) and Special Yeast and Mould Medium (SYM) at 35 ± 1 °C for 5 days. All colonies appearing at the end of incubation were counted and the results expressed as colony forming units per gram of honey (cfu/g).

Nutrient Agar (NA) is used for the cultivation of bacteria and for the enumeration of organisms (Difco™ & BBL™ Manual, 2009). It is a general medium recommended for the growth of a wide variety of nonfastidious microorganism such as *Escherichia coli* or *Pseudomonas aeruginosa*.

Special Yeast and Mould Medium (SYM) is used for isolating and cultivating yeasts and moulds. SYM is a nutritionally rich medium that supports the growth of a wide variety of yeasts. In addition, 0.1 g of chloramphenicol was added to the medium to inhibit the growth of organisms other than yeasts and moulds.

6.2.3. Antimicrobial Activity of honey

To determine the antimicrobial efficacy of honey, *Candida amapae* (yeast), *Saccharomyces cerevisiae* (yeast), *Escherichia coli* (gram -) and *Bacillus cereus* (gram +) were selected.

The microorganisms were obtained from the Spanish-type culture collection (CECT, Valencia).

       Escherichia coli was cultured aerobically at 37 °C in Nutrient Agar (NA) for 24 h, and it was then subcultured in liquid medium (Nutrient Medium) for 24 h at 37 °C, with continuous agitation. For experimental use, the microorganism was subcultured in liquid media (Nutrient Medium), incubated for 24 h at 37 °C, with continuous agitation (Rotabit orbital shaker incubator

J.P Selecta, Barcelona.) and used as the source of inoculums for each experiment. The concentration of the inoculum was measured spectrophotometrically (BioTek multi-reader detector, Germany) at 660 nm to be 1×10^3 and 1×10^6 colony forming units. To this purpose, McFarland Standards were prepared by preparing a 1% solution of anhydrous $BaCl_2$ and a 1% solution of H_2SO_4 and mixing them in different proportions. Although the McFarland Scale is designed to be used for estimating concentrations of gram negative bacteria, such as *E.coli*, it was also used for the rest of the microorganisms since the counts were confirmed then by inoculating the suspensions in plates containing agar medium when possible.

It was proceed likewise for the rest of the microorganisms with the required growth conditions for each microorganism.

Bacillus cereus coli was cultured aerobically at 30 °C in Nutrient Agar (NA) for 48 h, and it was then subcultured in liquid medium (Nutrient Medium) for 48 h at 30 °C, with continuous agitation.

C.amapae was cultured aerobically at 25 °C in Glucose Peptone Yeast Agar (GPYA) for 48 h. For experimental use, the microorganism was subcultured in liquid media (Glucose Peptone Yeast Medium), incubated for 48 h at 25 °C, with continuous agitation and used as the source of inoculums for each experiment. The turbidity of the yeast cell suspension was then adjusted to 0.5 on the McFarland scale (1×10^3 and 1×10^6 CFU/mL) with the sterile saline.

S. cerevisiae was cultured aerobically at 37 °C in Special Yeast and Mould Agar (SYM) for 48 h. It was then subcultured in liquid medium (Special Yeast and Mould Medium) for 48 h at 37 °C, with continuous agitation.

For the test assays, two concentrations of honey were used; the first concentration (H1, 0.5g/mL) was prepared by dissolving 1 g of honey with a magnetic stirrer in 2 mL of distilled water. The second (H2, 1g/mL) was made by diluting 1 g of honey in 1 mL of distilled water. All samples were kept at 20 °C for one hour before the test assay. A 96 multiwell plate was used for the screening of the antimicrobial activity. Each well contained 100 µl of the corresponding growth medium, 50 µl of inoculum and 50 µl of honey or Buffer solution (control). The plate was measured at intervals of 1h during 24 h or 48h when required, at 25, 30 and 37 °C. Moreover, as a

control 200 µl of the diluted honey samples were placed alone and measured the absorbance for 24h. Each point of the growth curve represented is an average of 8 replicates.

The mediums were prepared according the specifications of CECT.

Nutrient Agar:

Beef extract: 5 g

Peptone: 10 g

NaCl: 5 g

Agar powder: 15 g

Distilled water: 1 L

pH 7.2, 37°C

Note: For *B. cereus* 10 mg of $MnSO_4 \cdot H_2O$ were added.

Special Yeast and Mould Medium (SYM):

Glucose: 20 g

Mycopeptone: 1 g

Yeast extract: 3 g

Malt extract: 20 g

Agar powder: 20 g

Distilled water: 1 L

Glucose Peptone Yeast Agar (GPYA):

Glucose: 40 g

Mycopeptone: 5 g

Yeast extract: 5 g

Agar powder: 20 g

Distilled water: 1 L

6.2.4. Determination of Hidroxymethylfurfural (HMF) content in honey by HPLC

Refer to Chapter 2 section 1.

6.3. Results and Discussion

6.3.1. Microbiological analysis

Microorganisms that survive in honey are those that withstand the concentrated sugar, acidity and other antimicrobial characters of honey (P.B. Olatain *et al.,* 2007).

Rosemary raw honey incubated in Nutrient Agar (NA), Glucose Peptone Yeast Agar (GPA) and Special Yeast and Mould Medium (SYM) mediums presented microbial contamination. This is reasonably because the moisture and temperature conditions had change onto favourable conditions that profit the microorganisms (i.e. yeast, spore-forming bacteria) to develop. The NA Petri dishes presented white creamy colonies that occupied the totality of the plates after 24 h as shown in Picture 19 (A). The SYM Petri dishes presented a mean value of 4 dark green colonies of fungi and yeast after 24 h that also occupied the totality of the plates within 48 h (Picture 19 (B)). Nevertheless, these values were far below the maximum permissible statutory level (CODEX STAN 12-1981). It is important to mention that honey itself did not presented any sign of contamination, however when incubated (that is, diluted) it occurred the microbial growth. Hence, it is desired to dope out whether an ultrasound treatment can reduce the contamination of honey once diluted.
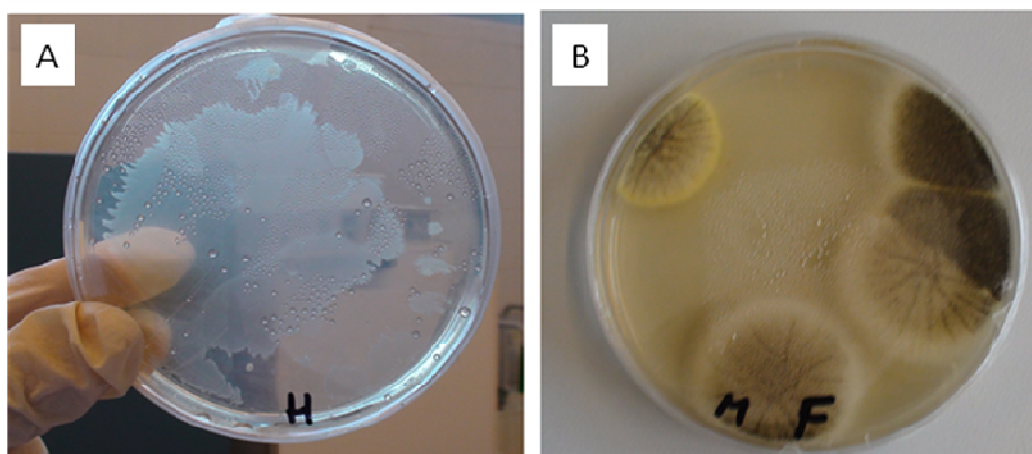
Picture 19. (A) honey incubated in Nutrient Agar. (B) Honey incubated in Special Yeast and Mould Medium.

Firstly, samples were sonicated (US sample) at the power level of 3 of the ultrasound bath (see M&M Chapter 3), at 40 °C for 20, 40 and 60 minutes and heat treated (HT sample) in the same conditions but without sonication, with regard the results of previous treatment conditions (Chapter 1 and 2). However, both samples (HT and US) presented high levels of microbial contamination. Surprisingly, it was found that the ultrasonicated (US) samples in NA presented higher contamination levels (113 cfu/g) than those not treated (87 cfu/g), and than the only heat treated (110 cfu/g). For the SYM cultures, the count was also higher. Picture 20 shows a representative illustration of the colony growth observed in SYM Petri dishes. Interestingly, as has occurred in NA plates, samples sonicated for 60 min (Picture 20 (C)) presented higher contamination than those treated for 40 min (Picture 20 (B)), and likewise samples sonicated for 40 min presented higher and bigger colonies than those treated for 20 min (Picture 20 (A)). As it can be observed, the longer the treatment time the bigger the colonies. These results can be justified by the fact that the temperature treatment (40 °C) was close to the optimal microbial temperature growth (37 °C) and short periods of sonication at this power and frequency (level 3, 40 kHz) cannot inhibit but promote the microbial expansion, as it could act as a stimulant.
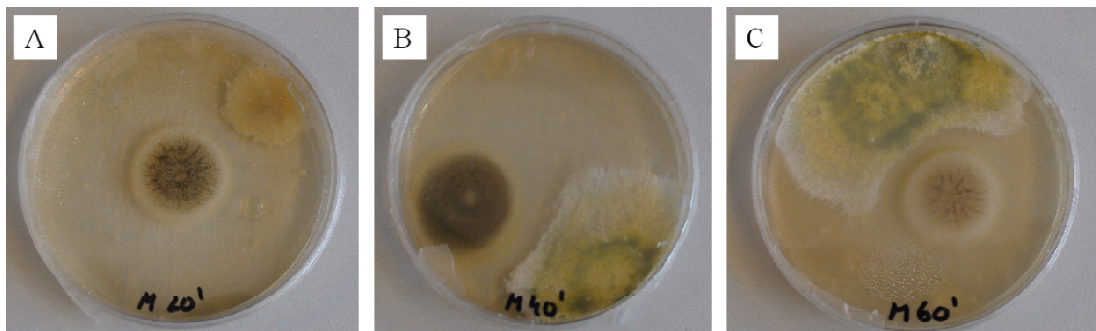
Picture 20. Colonies in SYM agar after 48 h. (A) Honey sonicated at 40 °C for 20 min. (B) Honey sonicated at 40 °C for 40 min. (C) Honey sonicated at 40 °C for 60 min.

The second trial performed was made by increasing the temperature treatment to 50 °C in order to overpass the optimal temperature growth. Levels of microbial contamination of honey samples are presented in Table 22. Samples were sonicated at 50 °C for 20, 40 and 60 minutes at level 3 (US samples), and other samples were only heat-treated in the same conditions but without

sonication (HT samples). However, even that both samples still presented microbial growth, the US samples showed a decrease of contamination (of 10 % approximately). Furthermore, comparing between the US samples, the treated ones for 60 minutes presented less contamination than those treated for 40 minutes. Likewise, the treated ones for 40 minutes presented less contamination than those treated for 20 minutes. These results lead to increase the time of treatment to emphasize the ultrasonic inhibition as the results were not significant.

Honey samples were sonicated at 50 °C for 90 and 120 minutes at the power level of 3 and heat-treated in the same conditions but without sonication. Samples sonicated for 90 minutes in NA showed a lower average of contamination (70 cfu/g) than the untreated ones (87 cfu/g) and the HT samples (78) cfu/g) after 48 h. This reduction though, is still not significant. For the SYM cultures, the sonicated samples presented an average of 2 colonies per gram of honey, while the HT presented an average of 4 colonies per gram (Picture 21). Moreover, the size of colonies of the US samples was considerably smaller. The US samples sonicated for 120 minutes in NA and SYM showed a significant decrease of microorganisms of 60 % (45 cfu/g) after 48 and 120 h. The US counts in SYM presented an average of just 1 colony per gram while the HT showed a mean value of 4 colonies per gram of treated honey. Likewise, the size of colonies in the ultrasonicated samples was noticeably smaller (Picture 22). For instance, the diameter of a colony in SYM agar plate measured 0.8 cm, while a colony in the heat treated sample measured 2.5 cm, after 48 h. This indicates that US delays the microbial growth increasing its lag phase.

Hence, from there on, the rest of experiments were at the test conditions at 50 °C for 2 h of treatment at the power level of 3 of the ultrasound bath.

Table 22. Count of microorganisms present in honey.

Sample			No. of samples	SYM CFU/mL			NA CFU/mL		
Treatment	t (min)	T (°C)		Range	Mean	Standard Deviation	Range	Mean	Standard Deviation
Raw honey			17	3.00-6.00	4.25	0.97	72.00-100.00	87.50	10.55
US	60	40	15	3.00-6.00	4.83	0.83	91.00-140.00	113.08	16.39
HT	60	40	15	3.00-6.00	4.67	0.78	95.00-135.00	110.00	11.87
US	90	50	15	1.00-4.00	2.58	1.08	56.00-80.00	70.00	8.44
HT	90	50	15	3.00-6.00	4.47	1.56	63.00-90.00	78.75	7.50
US	120	50	15	0.00-3.00	1.58	1.08	36.40-52.00	45.50	5.49
HT	120	50	15	7.00-2.00	4.08	1.56	62.72-89.60	78.40	9.46

SYM: Special Yeast and Mould Medium is used for isolating and cultivating yeasts and moulds.

NA: Nutrient Agar is a general medium for the enumeration of a wide variety of microorganisms.

US: Ultrasonicated sample.

HT: Heat-treated sample.
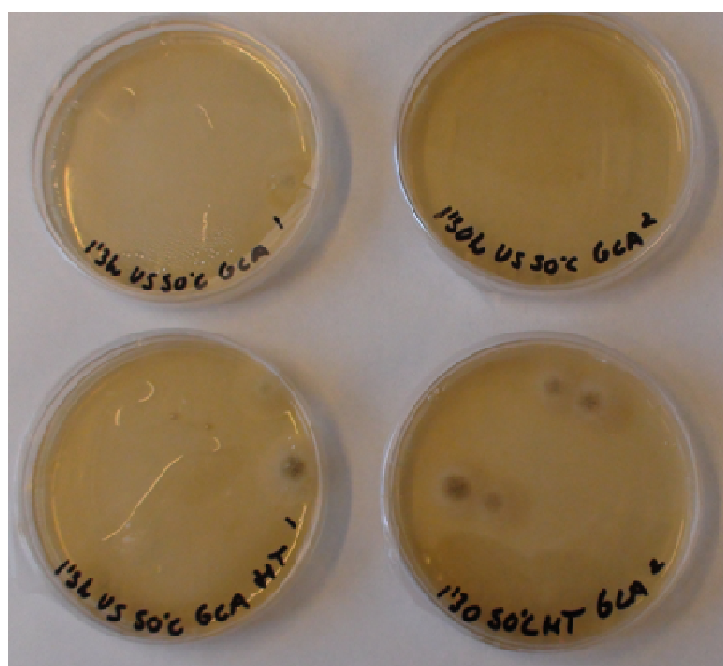
Picture 21. First row: US honey treated at 50 °C for 90 min incubated in GCA agar after 48h. Second row: HT honey at 50 °C for 90 min incubated in GCA agar after 48h.
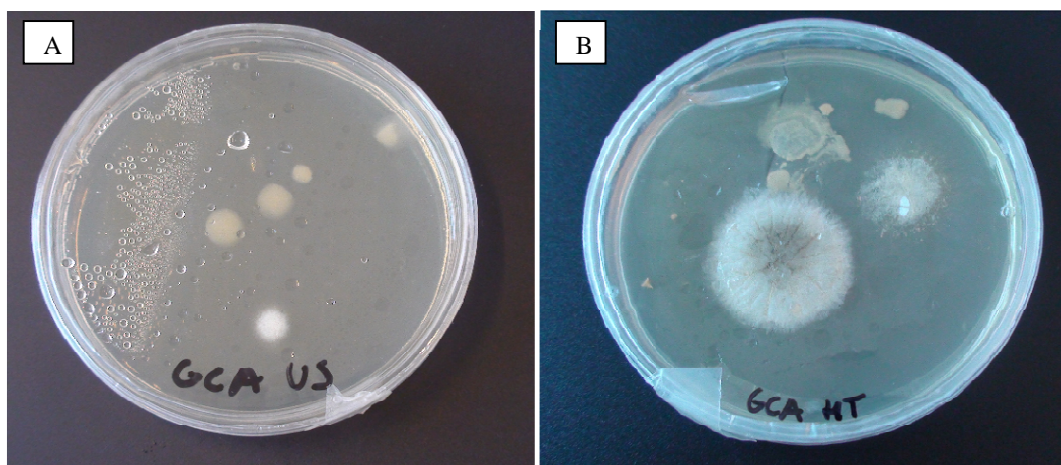
Picture 22. (A) us sample treated at 50 °C for 120 minutes incubated in GCA agar after 48 h. (B) ht sample treated at 50 °C for 120 minutes incubated in GCA agar after 48 h

6.3.2. Antimicrobial activity of honey

Honey has been reported to have a potent antibacterial activity, effective against a broad spectrum of species, and to have antifungal properties as well (E. Crane, 1983; P. Molan, 1992, J. A. Snowdon and D. O. Cliver, 1996; I. Escriche *et al.*, 2009). The antimicrobial activity of honey is attributed to some physical (acidity, osmolarity, a_w) and chemical (nectar, pollen) factors. When diluted, honey also shows an antimicrobial capacity due to hydrogen peroxide, a bacteriostatic agent produced by enzymatic activity. This enzyme is called glucose-oxidase and it is secreted from the hypopharyngeal gland of the bee into the nectar to help formulate honey from nectar. It is believed that the hydrogen peroxide is used as a sterilizing agent during the honey's ripening process. Glucose-oxidase has been found to be practically inactive in full-strength honey; it giving to raise hydrogen peroxide only when the honey is diluted (N. Altman, 2010). In fact, on dilution of honey, the activity increases by a factor of 2500 to 50.000, thus giving "slow-release" antiseptics at a level, which is antibacterial but not tissue damage (P.B. Olatain *et al.,* 2007). Interestingly, it has been shown that the hydrogen peroxide produced by honey is often better at killing bacteria than hydrogen peroxide obtained from other sources. It is thought that this is due to the fact that other substances present in honey (i.e phenolic compounds) seem to increase its bacteria-killing effect (L. Chepulis, 2008).

As it has been previously demonstrated in Chapter 2 (refer to section DN), ultrasounds did not affect the activity of the diastase enzyme. In this Chapter it is aimed at investigating the effect of US on glucose-oxidase enzyme activity. To determine the antimicrobial efficacy of honey,

several types of microorganisms were tested; *Candida amapae*, *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus cereus.* For all the assays honey samples (US and HT) were treated at 50 °C for 2 h.

Summary of the experimental conditions:

Tests carried out with *Escherichia coli*

E.coli is a gram-negative, facultative anaerobic and non-sporulating microorganism. *E.coli* counts in honey are a specific indicator of fecal contamination and its presence has rarely been reported in honey (J. A. Snowdon and D. O. Cliver, 1996). However, this microorganism can live on a wide variety of substrates since it has a great capacity to adapt to any adverse environment.

E.coli was prepared as explained in Material and Methods section (refer to Materials and Methods Chapter 3).

Microbial concentration of 10^6 CFU/mL

Figure 11 (A) shows the microbial growth of *E.coli* (1×10^6 CFU/mL) in US, HT and raw honey H1 (1g/2mL). The error bars shown at each measure indicate the great confidence among the replicates (8 measures each point) and indicate that the differences among the honey samples are statistically significant. As commonly interpreted, turbidity is an indicator of microbial

concentration; the higher the absorbance the higher the microbial development. The first aspect to consider in the graphic is that the sonicated honey presented always the lowest initial absorbance, which indicates that is more clarified than the HT and the raw honey. The second thing to note is that the absorbance increase in the US sample is less pronounced among the samples (HT and raw), suggesting that the inhibition effect on *E.coli's* growth is higher than the HT sample (Figure 11 (A)). Raw honey sample did not present an inhibition effect at all. This behaviour is also seen in Figure 11(B) with the honey more concentrated H2 (1g/1mL), which as before, the US honey had the lowest initial absorbance compared to the HT and the raw honey. Moreover, the inhibition of *E.coli* in the US sample is more pronounced at this concentration.

For all that, to better observe the inhibitory effect of rosemary honey, all curves were normalized so that they all started at the point 0, and thus the increase of *E.coli* over time is represented.

Figure 11 (C) shows the increase of absorbance of *E.coli* (1×10^6 CFU/mL) in US, HT and raw honey (H1 concentration). As it can be observed, the raw honey sample showed the highest rise, demonstrating no inhibition effect at all, followed by the HT sample, whereas the US treated sample showed notably the lowest increment. Figure 11(D) shows the normalized absorbance of the more concentrate honey samples (H2). *E.coli* is clearly more sensitive to the sonicated honey when more concentrated. The inhibitory effect of the US sample was found to be higher than that of the HT and raw honey samples. This could be because of the combined work of the US treatment and the glucose-oxidase activity, which activity is increased once diluted.

Figure 11. (A) *E.coli* 10^6 **CFU/mL in (●) US honey (▲) HT honey, and (■) raw honey at a concentration of H1 (1g/2mL). (B)** *E.coli* 10^6 **CFU/mL in honey H1 normalized growth curve. (C)** *E.coli* 10^6 **CFU/mL in honey H2 (1g/1mL). (D)** *E.coli* 10^6 **CFU/mL in honey H2 normalized growth curve.**

Microbial concentration 10³ CFU/mL

Figure 12 (A) shows the microbial growth of *E.coli* (1×10^3 CFU/mL) in US, HT and raw honey samples at 1g/2mL concentration (H1). Once more, the US sample presented the lower initial absorbance among the samples due to its low crystal content. Moreover, the US honey sample curve started its rise quite later (at 6 h) than the HT and raw honey ones (both at 2 h approximately), indicating that the effect of ultrasounds increases the lag phase of the microbial growth. Although the graphic may lead to interpret the US exhibited the lowest increase, the normalized Figure 12(C) shows that the microbial growth of the US was in the sample intensity as the HT and raw honey samples. However, the graph also confirmed the growth retardation, which indicates that an US treatment delays the microbial growth increasing its stationary phase.

As for the more concentrated honey H2 (1g/1mL), the same hindrance effect in the US sample is observed in Figure 12(B). The ultrasound-treated sample presented initially the lowest absorbance among the samples indicating to be the clearest one. The absorbance heightens, that is, the microbial growth was produced after 11 h of incubation, while the HT and raw honey started the exponential phase within the first hours of incubation. However, in this case, the final absorbance value of the US and HT were close to each other (although being the US below the HT sample), while the raw honey kept its rise. The normalized graphic shown in Figure 12 (D) confirmed this viewpoint.

These results agree with other authors who had investigated the antimicrobial effect of honey on *E.coli*. H.Selçuk and K. Nevin (2002) tested the antimicrobial effects of different Turkish honey samples by agar well diffusion method on clinically isolated bacterial species in media containing various concentrations of honey. Their study confirmed the antibacterial effect on several gram-negative bacteria. Most of the honey samples analyzed were inhibitory at 50% and above concentrations on *E.coli*. P. Molan (1992) also reported *E.coli* to be inhibited by different types of honey. He even reviewed that *E.coli* is inhibited by artificial honey (38.4% fructose and 30.3% glucose dissolved in sterile water) when diluted 1 in 10 due to the osmotic effect. K. Brudzynski and L. Kim (2011) analysed Canadian honeys from diverse floral sources and were also screened against *Escherichia coli* (1×10^6 CFU/mL) using the broth microdilution method. Their results confirmed the inhibitory effect on *E.coli*. They also found that artificial honey (25% v/v) inhibited the microbial growth due to honey osmolarity. T.T. Adebolu (2005) evaluated the antimicrobial activity of two Nigerian honeys some bacterial species that cause diarrhea by agar well diffusion method. *E.coli* was found to be inhibited in a concentration of 1:8 of honey in sterile distilled water (v/v) for both honey samples. J. M. Wilkinson and H.M.A. Cavanagh (2005) determined the antibacterial activity of 13 Australian honeys, including 3 commercial ones, using standard well diffusion methods. All honeys, and an artificial honey,

were tested at four concentrations (10%, 5%, 2.5%, and 1% wt/v) against *E.coli* and zones of inhibition were measured. All honeys tested had an inhibitory effect on the growth of *E.coli* with one honey still having activity against *E.coli* at 2.5%, but no honey was active at 1% concentrations. C. Basualdo *et al.*,(2007) carried out a study to determine the antibacterial activity of 10 honeys produced in Argentina. However, the authors only found inhibitory effect in 4 of 10 of the undiluted honey samples, and no inhibitory effect in undiluted honey at 50 of 75%. D.P. Mohapatra *et al.*, (2010) evaluated the in vitro antibacterial activity of methanol, ethanol, and ethyl acetate extracts of raw and processed honey against gram-positive and negative bacteria (1×10^8 CFU/mL) by broth dilution technique. The authors reported that both types of honey showed antibacterial activity against the tested organisms. Gram-negative bacteria were found to be more susceptible as compared to Gram-positive bacteria. Among all the extracts analyzed, the authors found the methanol extract to be the most effective as an antibacterial agent.

As P. Molan (1992) pointed out, the major differences in findings on the sensitivity of each bacterial species are more likely to be due to differences in the honeys used. Many authors have demonstrated that not all honey samples have the same degree of antibacterial activity; therefore the sensitivity of species cannot be compared using the results from different studies, as the honeys used in the studies may have had widely differing antibacterial activity. The sensitivity of species relative to each other can be validly determined within a single study in which the same honey and same test conditions are used. Even so, the relative sensitivity of species could be found to be different within another study because species could respond differently to the different types of antibacterial factor that may be present in a different honey.
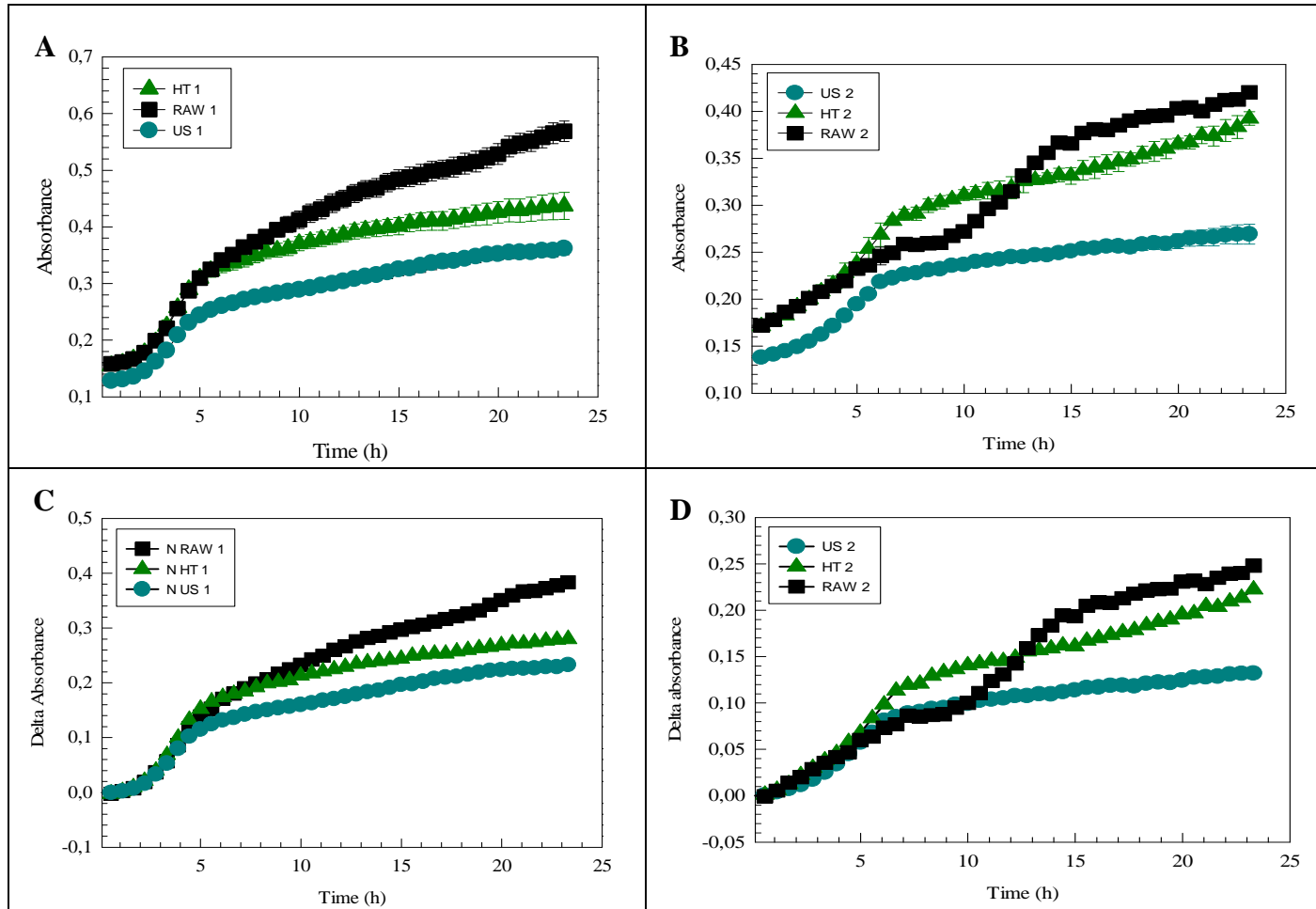
Figure 12. (A) *E.coli* 10^3 CFU/mL in (●) US honey (▲) HT honey, and (■) raw honey at a concentration of H1 (1g/2mL). **(B)** *E.coli* 10^3 CFU/mL in honey H1 normalized growth curve. **(C)** *E.coli* 10^6 CFU/mL in honey H2 (1g/1mL). **(D)** *E.coli* 10^6 CFU/mL in honey H2 normalized growth curve.

Tests carried out with *Candida amapae*

Honey contains naturally different osmotolerant yeast, which can cause undesirable fermentation.

Candida is a dimorphic fungus, i.e. it can take two forms. Most of the time it exists as oval, single yeast cells, which reproduce by budding. There are many species of the genus *Candida* which cause the disease known as candidiasis or "thrush". *Candida* is a commensal organism found in 40-80 % of normal humans, and is present in the mouth, gut, and vagina. The incidence of fungal infections is increasing in community and hospital environments (C.U. Anyanwu, 2012) and no other mycotic pathogen produces as great a spectrum of opportunistic diseases in humans and animals as *Candida* does. (P.G. Pappas *et al.*, 2003).

Most studies of honey antifungal activity are performed with *Candida albicans*, as this yeast is the   origin of   most oral   and   genital infections in   humans, and   hence the   interest in inhibiting its growth. However, the rate of candidaemia caused by non-albicans species is increasing. (M.L Estevinho *et al.,* 2011).

According to the Spanish Royal Decree 664/1997 *Candida albicans* yeast is classified as risk 2, which is defined as "*biological agent that can cause disease in humans and may pose a danger to   workers,   making   it   unlikely to   spread   to the   community   and having   usually effective prophylaxis or treatment to cure it*".

To work with a microorganism of risk 2 it is required to hold a number of special requirements that did not allow its manipulation in the College premises, and therefore it was resorted to Article 5 of the Royal Decree which states that "*given the technical and scientific information available, the employer, when the nature of the activity so permits, must avoid the use of dangerous biological agents through their replacement by other agents, depending on the conditions of use to be safe for the health of workers, or are less so*".

For all the above mentioned, *Candida albicans* was preplaced by *Candida amapae*, which is a microorganism of risk 1. Moreover, there does not appear to be significant difference in pathogenic potential of different *Candida* strains, therefore establishment of infection appears to be determined by host factors and not the organism itself (MicrobiologyBytes, nd).

C. amapae was prepared as explained in Material and Methods of Chapter 3.

Figure 13 and 14 shows the microbial growth of *C. amapae* in US, HT and raw honey samples. The error bars shown at each measure can hardly be observed in the graph due to the high reproducibility of the measures, which gives great confidence among the replicates (10 measures each point) and indicate that the differences among the honey samples are statistically significant.

Microbial concentration of 10^6 CFU/mL

Figure 13 (A) shows the microbial growth of *C. amapae* (1×10^6 CFU/mL) in presence of US, HT and raw honey at 1g/2mL concentration (H1). As it can be seen, the US treated sample initially presented the lowest absorbance among the samples, indicating to be the most limpid one. Moreover, the US sample presented a flat curve suggesting that no microbial growth occurred within the first 35 hours. The HT sample presented a slight increase within the 10 first hours, while the raw honey exhibited the sharpest increase in the initial 10 h of incubation and kept its increase after 20 h. Figure 13 (C) shows the absorbance normalized curve of *C. amapae* (1×10^6 CFU/mL) in presence of US, HT and raw sample honey H1 (1g/2mL). It is confirmed that raw honey did not present an inhibition effect at all, while the HT sample exhibited a slight but progressive increase in absorbance. This could mean that *C. amapae* is inhibited to some extent but its development still occurs within the well. The sonicated sample showed a flat tend curve up to 35 h. There was then a gradual increase suggesting that *C. amapae* started its development in the well, probably because the action of glucose-oxidase had had been reduced.

Figure 13(B) shows the microbial growth of *C. amapae* (1×10^6 CFU/mL) of concentrated honey sample H2 (1g/1mL). Once more, the US sample manifests to be the clearest one, as it started with the lowest absorbance. The HT and raw honey in this case presented almost the same absorbance. Both samples also exhibited a slight and progressive raise; however the raw honey sample displayed a sharp increase after 30 h of incubation, whereas the HT sample remained its slight increase. The US sample did not present any increase, which means *C. amapae* growth was inhibited. Figure 13(D) shows the normalized absorbance curves of *C. amapae* (1×10^6 CFU/mL) in presence of concentrated honey sample H2 (1g/1mL). The graph shows that all sample presented an initial increase within the first 10 h. However, in the sonicated sample the growing stabilized after 10 h while the HT sample continued a progressive increase. The raw honey sample also exhibited a straight up increase after 30 h of incubation. It is then deduced that the highest inhibition effect was again observed in the sonicated sample.
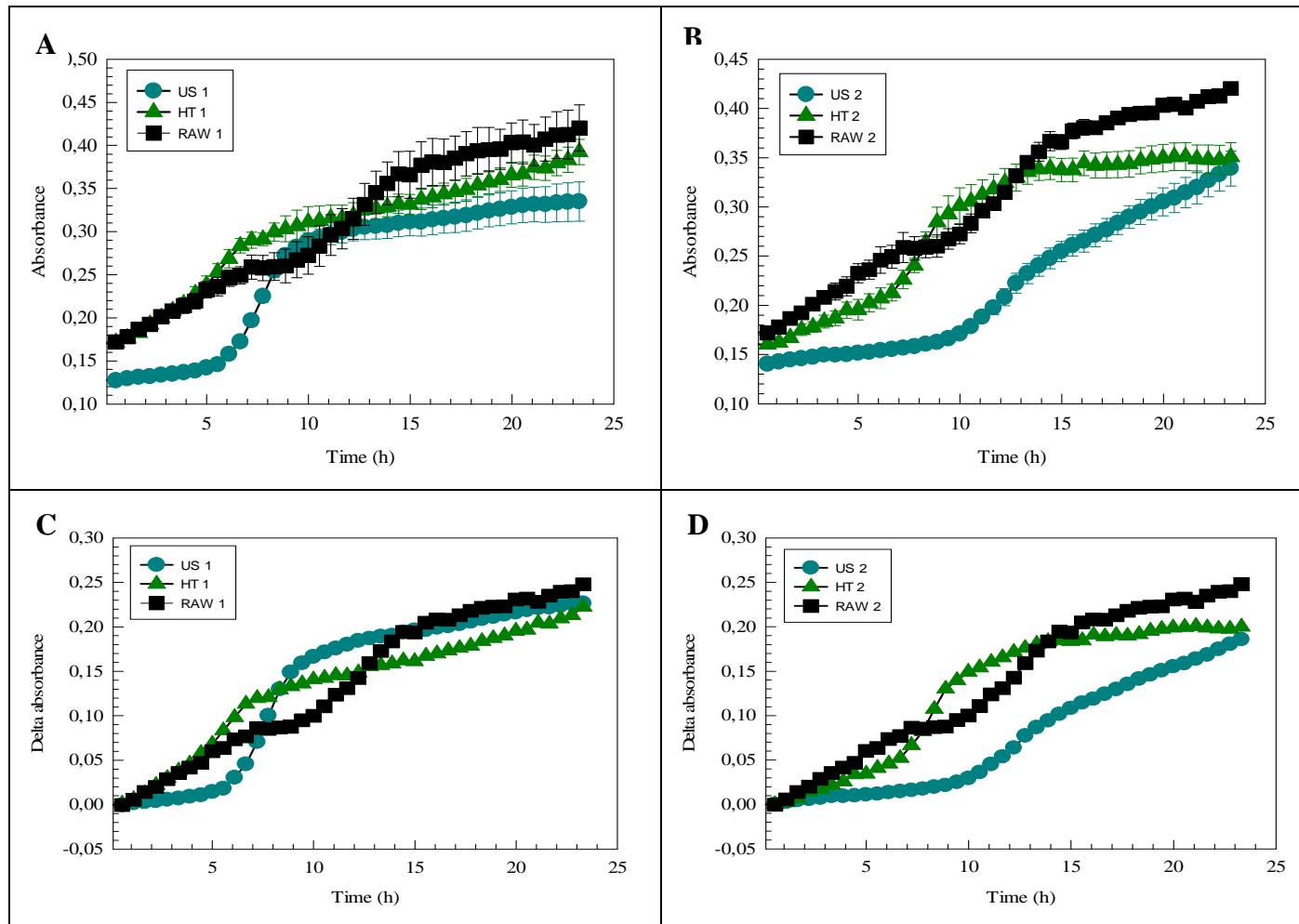
Figure 13. (A) *C. amapae* 10⁶ CFU/mL in (●) US honey (▲) HT honey, and (■) raw honey at a concentration of H1 (1g/2mL). (B) *C. amapae* 10⁶ CFU/mL in honey H1 normalized growth curve. (C) *C. amapae* 10⁶ CFU/mL in honey H2 (1g/1mL). (D) *C. amapae* 10⁶ CFU/mL in honey H2 normalized growth curve.

Figure 14(A) shows the microbial growth of *C. amapae* (1×10^3 CFU/mL) in presence of US, HT and raw honey at 1g/2mL concentration (sample H1). As the graph shows, there is a clear difference between the starting point and the final absorbance value among the samples. Thus, the sonicated sample (US) presented the lowest absorbance from the beginning and throughout the 48 hours of incubation, implying the inhibition of *C. amapae*, whereas the heat-treated sample (HT) showed a steady and soft raise. It is interesting to note the peaks observed after 30 h of incubation. The spectrophotometrical evaluation of the inhibitory effect of rosemary honey on *Candida amapae* had inherent problems. *C. amapae* is a yeast that develops from 20-48 h of incubation. Moreover, its growth in the well is first produced in the vicinity of the well and then expands to its centre. The spectrophotometer light beam crosses the plate through the centre of each one of the wells; it is therefore that in some cases, the growth is not reflected in the absorbance reading, although being occurred, and hence the observed peaks. The same fact occurs in the raw honey sample, the same peaks are observed after 30 h of incubation. *Candida*'s growth was clearly visible in the well, although in some measures the absorbance seemed minor. Figure 14(C) shows the growth increase of *Candida amapae* in presence of US, HT and raw honey samples H1 (1g/2mL). As noted, raw honey did not presented inhibitory effect on *C. amapae* whereas the heat-treated sample hindered the development of *C.amapae* to some extent. The curve of the ultrasonicated honey sample exhibited a small incline in the first 5 hours and then remained flat, which means that *C.amapae* was inhibited.

Figure 14(B) represents the yeast growth of *C.amapae* in presence of US, HT and raw honey H2 (1g/1mL). Once again is confirmed that the sonicated sample started with the lowest absorbance reading, which means is the most translucent among the samples, whereas the HT and raw honey presented almost the same starting absorbance. It could be deduced a microbial development in the sonicated sample since an increase of absorbance was observed within the first 10 hours. However, this growth cannot be attributed to a yeast development because *C.amapae* does not evolve until 20 h of incubation, and that time, the curve was shown flat, which means the yeast development was inhibited. On the other hand, the HT and raw honey samples showed a marked growth from the incubation commence, and it continued after 30 h, which indicates the development of *C.amapae*.

Figure 14 (D) shows the normalized absorbance curve of *C.amapae* in US, HT and raw honey at 1g/1mL concentration (H2). As mentioned before, the sonicated sample exhibited an increase on the first 10-15 h and then remained flat, implying the yeast inhibition. The heat-treated honey sample however exhibited a sharpest increase within the 20 first hours and then it another escalation was observed between the thirtieth and fortieth hours of incubation. Once more, the raw honey sample did not manifest an inhibition effect.
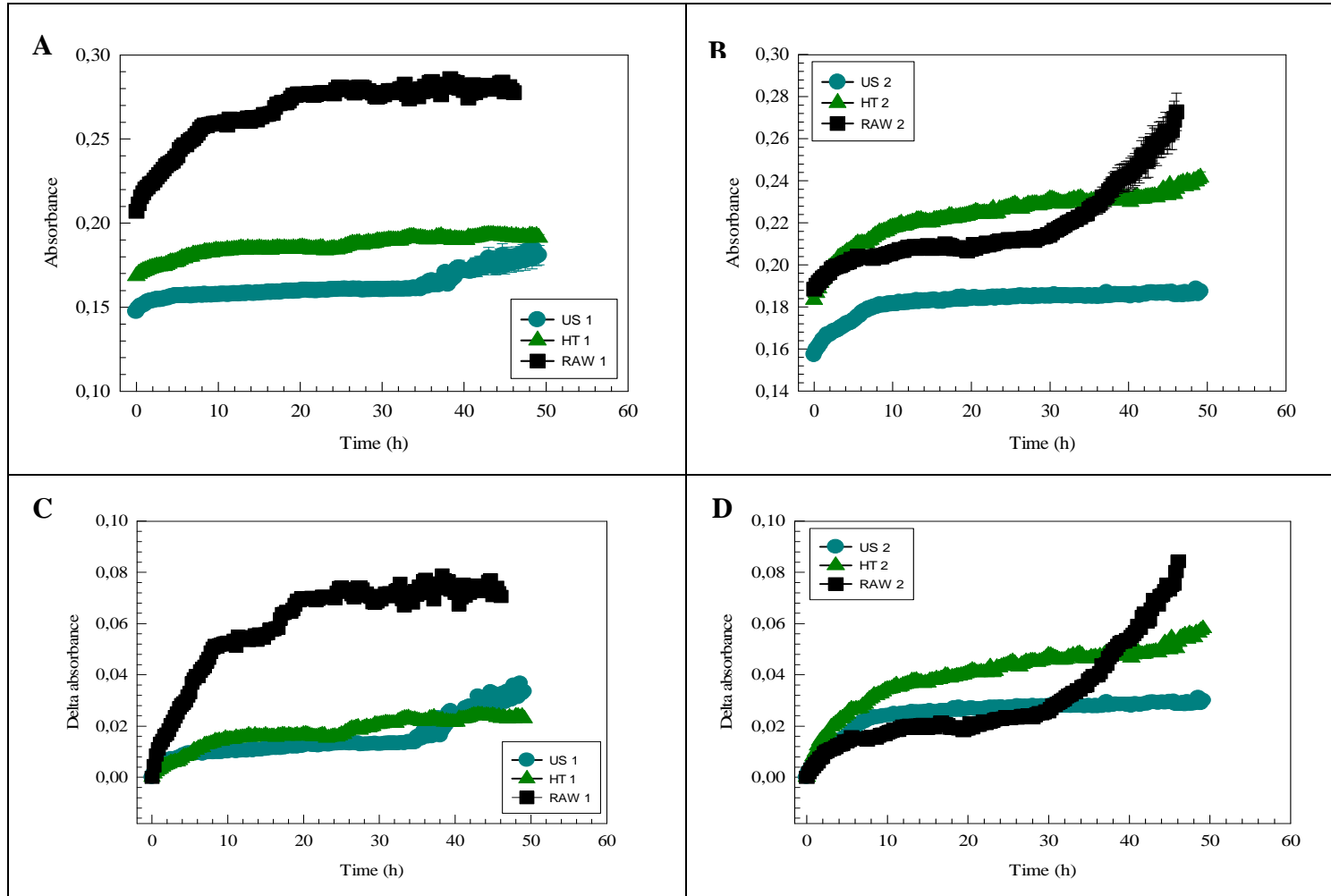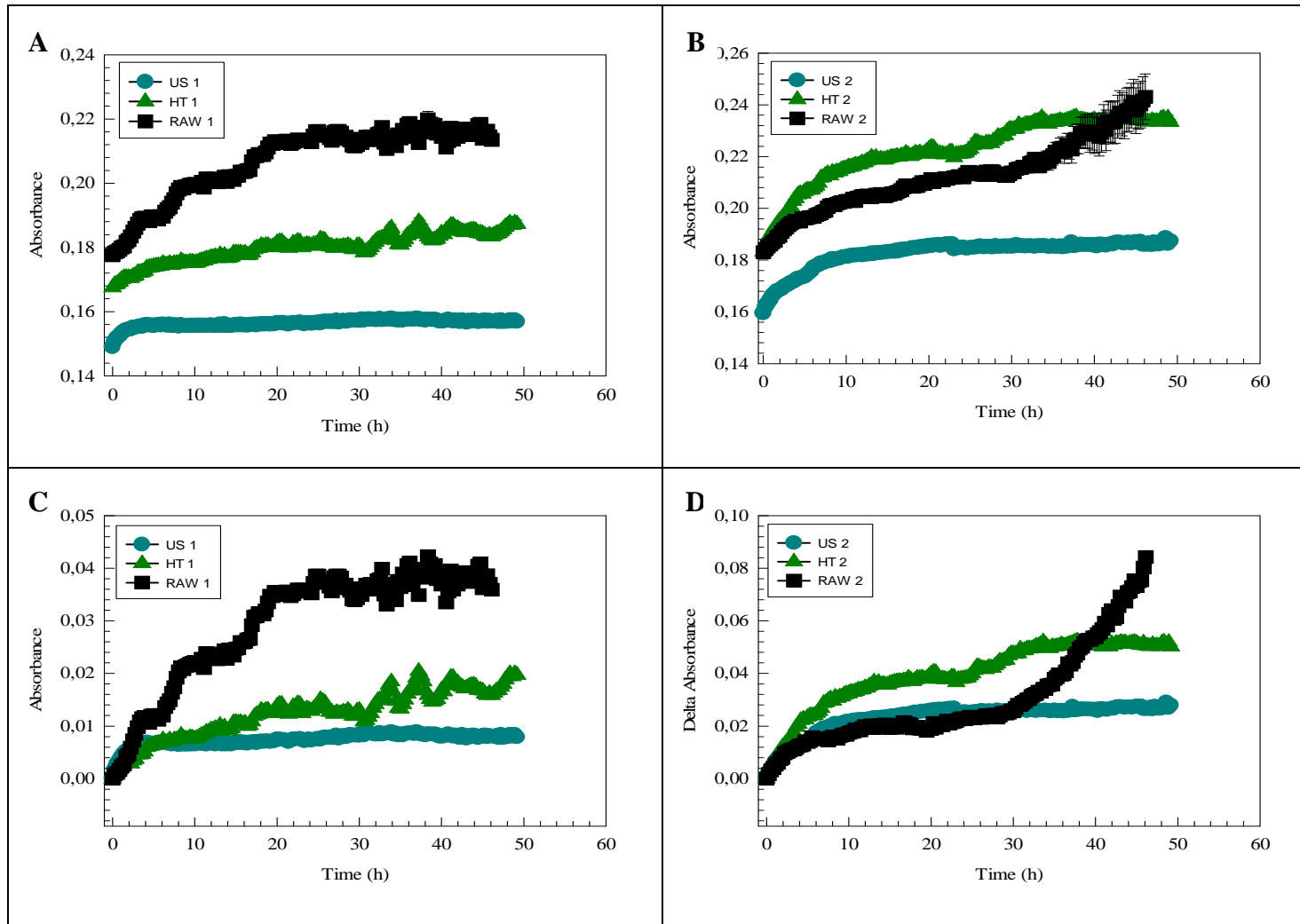
Figure 14. (A) *C. amapae* 10^3 CFU/mL in (●) US honey (▲) HT honey, and (■) raw honey at a concentration of H1 (1g/2mL). (B) *C. amapae* 10^3 CFU/mL in honey H1 normalized growth curve. (C) *C. amapae* 10^3 CFU/mL in honey H2 (1g/1mL). (D) *C. amapae* 10^3 CFU/mL in honey H2 normalized growth curve.

A number of previous studies have demonstrated that various honeys, both commercially and locally produced have antibacterial and antifungal activity. P.E. Lubsy *et al.* (2005) performed an agar dilution method to assess the activity of Australian honeys against 13 bacteria and one yeast (*Candida albicans*). The honeys were tested at five concentrations ranging from 0.1 to 20% (v/v). Little or no antibacterial activity was seen at honey concentrations below 1%, with minimal inhibition at 5%, however the greatest inhibition was seen at 20%. *C. albicans* was only inhibited at concentrations of 20%. In contrast of these authors, T. Ijaz *et al.* (2008) who also performed an agar dilution method reported the yeast *Candida albicans* was not inhibited by the Pakistani honeys at 20%. Nzeako and Hamdi (2000) in their study (by agar dilution) of six commercial Saudi-Arabian honeys found that honey inhibited *C. albicans* at honey concentrations higher than 40%, although the zone of inhibition was small compared with other organisms. Koc *et al.* (2009) evaluated the effect of Turkish honey samples from different floral sources for their ability to inhibit the growth of 40 yeast strains (*Candida albicans, C. krusei, C. glabrata and Trichosoporon spp.*). Broth microdilution method was used to assess the activity of the honeys against yeasts at different concentrations ranging from 1.25-80 % (v/v). All of the yeast strains tested were inhibited by honeys in their study. Broth microdilution assay revealed that inhibition of growth depends on the type and concentration of honey as well as the test pathogen. The study of M.L.Estevinho *et al.* (2011) assessed the in vitro antifungal properties of Portuguese lavender honey (10-60 % (v/v)) against *Candida albicans, Candida krusei*, and *Cryptococcus neoformans*. The results showed that the increase of lavender honey concentrations caused a decreased in the specific growth rate of all organisms studied. Values ranged from 31.0% (*C. albicans*), 16.8% (*C. krusei*) and 23.0% (*C. neoformans*); *C. krusei* was the most susceptible to honey since growth inhibition is reached at the minor level. M. Kuçut *et al.* (2007) performed a study designed to assess the in vitro biological activities of three Turkish honey samples (Chestnut, Heterofloral and Rhododendron). A simple susceptibility screening test, using the agar-well diffusion method, was employed against *C. albicans and C. tropicalis* (1×10^6 CFU/mL) among others microorganisms. Antimicrobial activities of the honeys were tested by using methanolic extracts of 50% concentration. Two honeys presented moderate inhibition against *Candida* species whereas one type did not show inhibition effect at all (Rhododendron).

It is noteworthy that honey concentrations vary greatly from one study to another. Part of the explanation for the diversity in concentrations may be due to methodological differences between studies, the type (composition) of honey being used and the microorganisms tested. Usually, studies performed by agar diffusion use lower concentrations, while those of broth microdilution usually employ higher. This is probably because microorganisms in the

liquid broth are more active because they have more mobility and there is more diffusion of nutrients, so they are more likely to develop than if they remain trapped in the agar. Moreover, the microbial number, as seen, also plays a crucial role in development.

In this study it has been used the broth microdilution method to assess spectrophotometrically antibacterial and antifungal activity rather than agar well diffusion that is the most common method for testing honeys. In broth microdilution methods the honey is incorporated directly into the growth media; hence, the microorganisms are brought into direct contact with all honey components immediately on application to the well rather than relying on diffusion of constituents through the agar.

Several factors may influence the antifungal activity of honey. For example, DeMera and Angert (2004) reported that honey from different phytogeographical regions varied in their ability to inhibit the growth of yeasts, suggesting that botanical origin plays an important role in influencing the antifungal activity. In addition, there are a great variety of components, including phenolic acids, flavonoids and other biomolecules, in different honeys. Biological activity of honey is mainly attributed to the phenolic compounds (L.M. Estevinho *et al.*, 2008).

Tests carried out with *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is an osmotolerant yeast which grow on high-moisture sugars. *Saccharomyces* species can be readily isolated from honey combs and adult bees (J. Snowdon, 1996), in fact *Saccharomyces spp*. represents the dominant yeast found in honey (C. Tysset and M. Rousseau, 1981).

However, honey's antibacterial factors are reported also to be effective against many fungal species, including *Aspergillus, Candida, Penicillium,* and *Saccharomyces* (J. Snowdon, 1996). Figures 15 and 16 shows the microbial growth of *S. cerevisiae* in presence of US, HT and raw honey samples. The error bars shown at each measure can hardly be observed in the graph due to the high reproducibility of the measures, which gives great confidence among the replicates (10 measures each point) and indicate that the differences among the honey samples are statistically significant.

Microbial concentration of 10^6 CFU/mL

Figure 15(A) shows the microbial growth of *S. cerevisiae* (1×10^6 CFU/mL) in US, HT and raw honey sample H1 (1g/2mL). Once more, the US sample presented clearly the lowest initial absorbance point among the samples. The less the absorbance the clearer the honey, which means that US treatment liquefies better the honey. Moreover, the sonicated sample curve showed the lowest development of culture growth, being *S. cerevisiae* affected to a greater extent, since the absorbance rise was the least heightened among the samples. The heat-treated sample did also present an inhibition effect since the growth of *S. cerevisiae* was also hindered

compared to the raw honey sample. Figure 15(C) represents the normalized curve of shows the microbial growth of *S. cerevisiae* (1×10^6 CFU/mL) in presence of US, HT and raw honey sample H1 (1g/2mL). As the graphic shows (Figure 17), the raw honey sample exhibited the highest absorbance's increase, followed by the HT sample and last the US treated sample. It can be also observed, that all samples presented the sharpest increase within the first 8 h of incubation, however, the sonicated sample remained flat afterward (which means the yeast development ceased), whereas *S. cerevisiae* in the HT sample kept on growing since the curve presented a progressive rise. So did the raw honey sample with a much larger increase. This could be because of the combined work of the US treatment and the glucose-oxidase activity, which activity is increased once diluted.

Figure 15(B) shows the microbial growth of *S. cerevisiae* (1×10^6 CFU/mL) in presence of US, HT and raw honey sample H2 (1g/1mL). The graph shows the same behaviour repeated in all assays; the US treated sample started with the minimum absorbance point, followed by the HT sample, whereas the raw honey sample started always with the highest absorbance among the samples. As for the inhibition effect, all samples showed yeast growth. *S. cerevisiae* was developed the most in raw honey, whereas US and HT samples demonstrated some inhibition response. Figure 15(D) shows the normalized curve of the microbial growth of *S. cerevisiae* (1×10^6 CFU/mL) in presence of US, HT and raw honey sample or concentration H2 (1g/1mL). These results confirms *S. cerevisiae* developed in all samples. Nonetheless, the curve of the sonicated sample showed the lowest development of culture growth, which was close below to the HT sample. Raw honey sample on the other hand, did not show any microbial inhibition on *S. cerevisiae.*
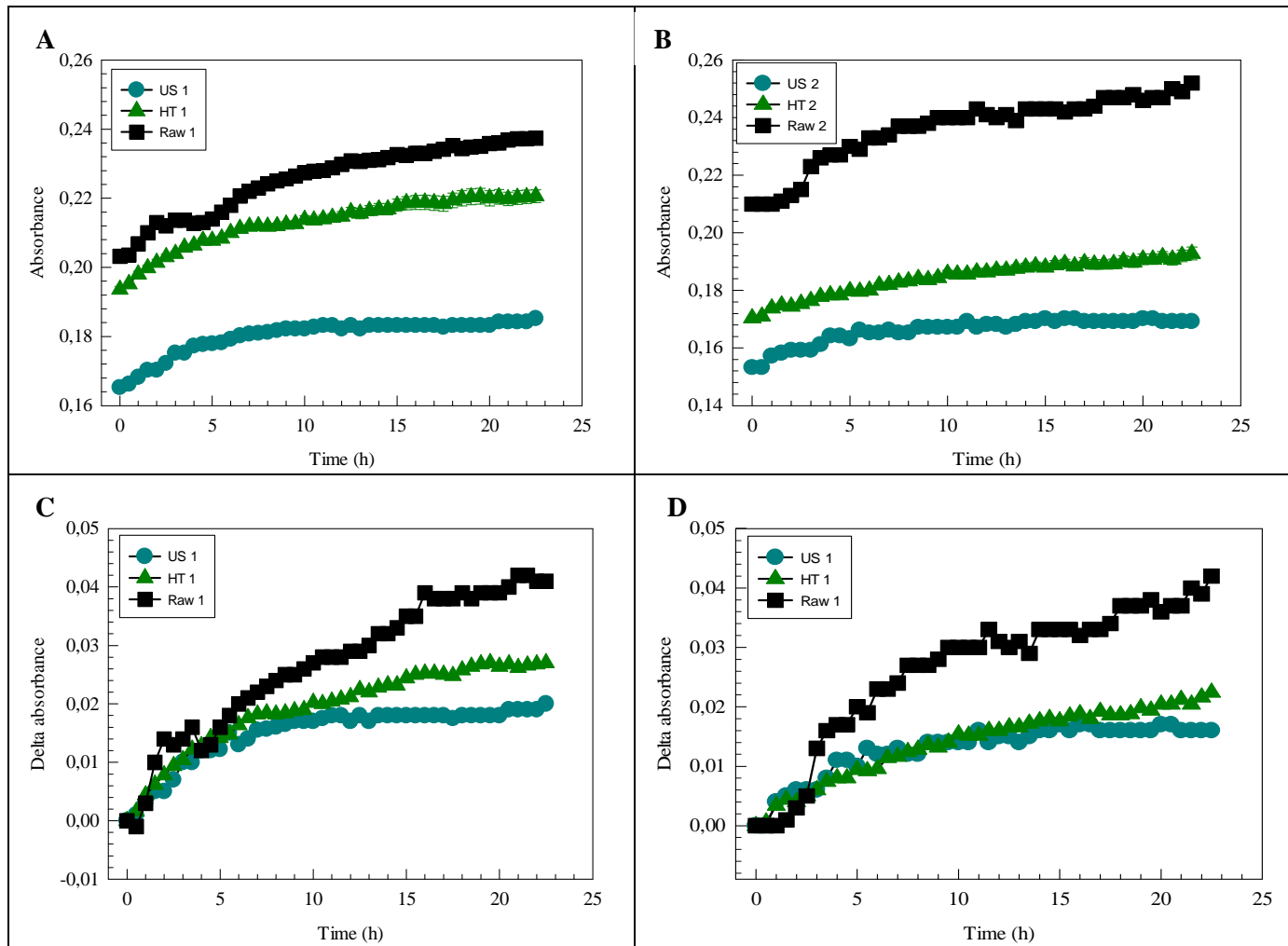
Figure 15. (A) *Saccharomyces cerevisiae* 10^6 CFU/mL in (●) US honey (▲) HT honey, and (■) raw honey at a concentration of H1 (1g/2mL). **(B)** *S. cerevisiae* 10^6 CFU/mL in honey H1 normalized growth curve. **(C)** *S. cerevisiae* 10^6 CFU/mL in honey H2 (1g/1mL). **(D)** *S. cerevisiae* 10^6 CFU/mL in honey H2 normalized growth curve.

Figure 16 shows the yeast growth of *S. cerevisiae* (1×10^3 CFU/mL) in US, HT and raw honey samples. The error bars are also represented although they can barely be perceived due to the high reproducibility of the spectrophotometric measurements. Reiteratively, the sonicated sample started with the minimum absorbance value, followed by the HT sample and raw honey samples, the latter one starting with the highest absorbance point.

Figure 16(A) shows the yeast growth of *S. cerevisiae* (1×10^3 CFU/mL) in presence of US, HT and raw honey concentration H1 (1g/2mL). As it can be observed *S. cerevisiae* developed in the US treated sample, in the first 3 hours of incubation, as an ascension in absorbance was observed. However, the yeast growth seemed to have stopped after that, since the curve is shown plain until the end of the incubation. Heat-treated and raw honey samples on the contrary, presented a continuous ascension through the end of the incubation period, indicating *S. cerevisiae* kept on growing in the well. Figure 16(C) shows the normalized curves of *S. cerevisiae* (1×10^3 CFU/mL) in presence of US, HT and raw honey H1 (1g/2mL). As the curves shown, all samples presented a rise in absorbance within the first hours of incubation; however, the sonicated sample hindered the yeast growth considering the flat tendency observed. *S. cerevisiae* was not affected by either the HT or raw honey samples, for the reason that both samples continued increasing its absorbance. In fact, at the end of the experiment, the sonicated sample presented the half absorbance rise of both HT and raw honey samples.

Figure 16(B) shows the yeast growth of *S. cerevisiae* (1×10^3 CFU/mL) in presence of US, HT and raw honey concentration H2 (1g/1mL). In this more concentrated in honey, however, the same behaviour is observed, the US treated sample inhibits the growth of *Saccharomyces* to a great extent, whereas both HT and raw samples do not affect its development. Figure 16(D) represents the normalized curves of the yeast growth of *S. cerevisiae* (1×10^3 CFU/mL) in presence of US, HT and raw honey sample H2 (1g/1mL). The heat-treated sample presented a delay in absorbance increase compared to the raw sample; however, the yeast carried forward its growth, even over passing the raw honey sample growth. The ultrasound-treated sample on the other hand, inhibited eminently the yeast growth. The final absorbance rise of the US sample was three times lower than HT and raw honey samples.

So far, there are now only few works published testing the antifungal activity of honey against *S. cerevisiae*. A. Laorpaksa *et al.* (1992) evaluated the antifungal activity of 10 types of Thai honey on *Candida albicans* and *Saccharomyces cerevisiae* by agar well diffusion method. The authors reported that none of the honey tested presented inhibition effect on these two microorganisms despite the concentration (10-50 %) used. S. Gomez *et al.* (2010) evaluated the antimicrobial effect of Portuguese honey against fermentative yeasts. Honey samples were pasteurized at 75 °C for 30 min prior to analysis. Yeast suspensions (approximately, 10^6

CFU/mL) were mixed in 0 %, 10 %, 25 % and 50 % of honey and made up to 25 mL with yeast broth. *S. cerevisiae* showed only a slight sensitivity to honey, being barely affected by the different honey concentrations tested. The authors suggested these results could be justified by the fact that *S. cerevisiae* yeast has been isolated from honey, so the yeast is probably adapted to the stress conditions found in honey. In fact, there are many studies evaluating the capacity of *Saccharomyces cerevisiae* strains, isolated from honey to produce mead, a traditional drink, which results from the alcoholic fermentation of diluted honey carried out by yeasts (M. Navratil *et al.*, 2001; A. P. Pereira *et al.*, 2009; A. Mendes-Ferreira *et al.*, 2010, among others).
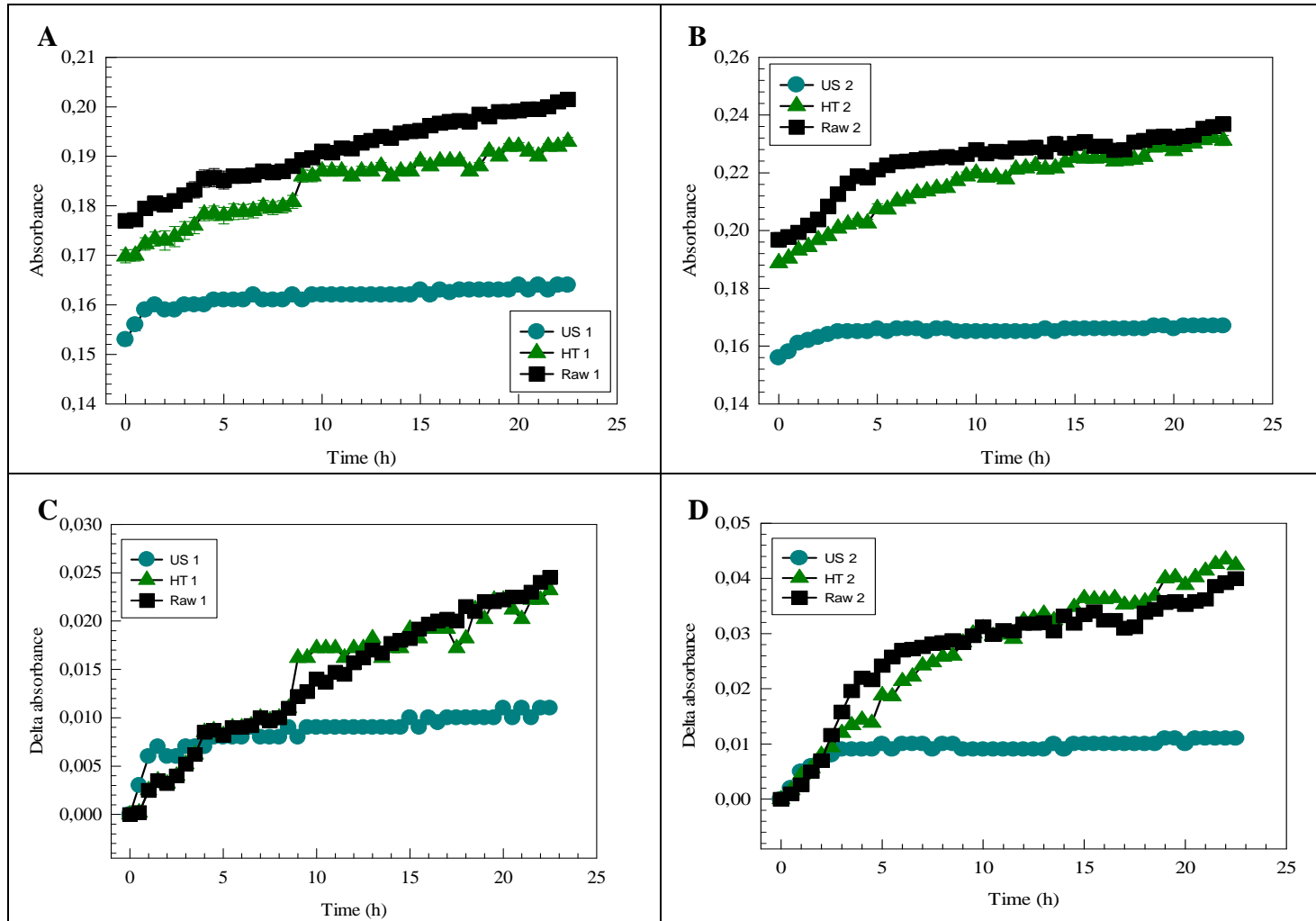
Figure 16. (A)) *Saccharomyces cerevisiae* 10^3 CFU/mL in (●) US honey (▲) HT honey, and (■) raw honey at a concentration of H1 (1g/2mL). **(B)** *S. cerevisiae* 10^3 CFU/mL in honey H1 normalized growth curve. **(C)** *S. cerevisiae* 10^3 CFU/mL in honey H2 (1g/1mL). **(D)** *S. cerevisiae* 10^3 CFU/mL in honey H2 normalized growth curve.

Tests carried out with *Bacillus cereus*

Microbial concentration of 10^6 CFU/mL

Bacillus cereus is a soil-dwelling, gram-positive rod-shaped bacterium. The bacterial spores of the Bacillus genus are regularly found in honey (J. A. Snowdon, 1996). Organisms found in the environment around honey (i.e. bees, hives, pollen, flowers, soil, etc.) are also likely to occur in honey, and Bacillus among others are bacteria commonly found in soil. Moreover, amidst the bacteria introduced to honey by the bees are found the members of the Bacillus family (C. Tysset and M. Rousseau, 1981).

Figure 17 and 18 shows the microbial growth of *Bacillus cereus* in US, HT and raw honey samples.

Figure 17(A) shows the microbial growth of *B. cereus* (1×10^6 CFU/mL) in US, HT and raw honey sample H1 (1g/2mL). As it can be observed, in agreement with the other studied samples, the sonicated honey sample started with the lowest absorbance value among the samples. Moreover, it also exhibited the lower increase of absorbance throughout the 48 h of the incubation period, whereas the heat-treated and raw honey samples showed the same trend in terms of microbial growth. Even though, none of the treatments inhibited completely the growth of the microorganism at this honey concentration, since all samples started the increase concurrently within the first 5 hours of incubation. Figure 17(C) represents the normalized absorbance curves of Figure 17(A), which confirmed the US treated sample depleted greatly the growth of *B. cereus*, whereas the HT and raw honey samples did not at all.

Figure 17(B) shows the microbial growth of *B. cereus* (1×10^6 CFU/mL) in presence of the concentrated honey samples H2 (1g/1mL). Although the sonicated sample presented the lowest absorbance value among the HT and raw honey samples, this difference is not as distant as the initial absorbance observed among the samples when tested in other microorganisms (i.e. *E. coli)*. Moreover, all samples exhibited an intense rise in absorbance within the first 5 h and it kept increasing till the end of the incubation period. Figure 17(D) represents the normalized absorbance curves of *B. cereus* (1×10^6 CFU/mL) in honey sample H2 (1g/1mL). From this graph (Figure 17), the different sample curves can be hardly distinguished, and thus no inhibitory effect is evidenced. The lack of inhibition effect in honey H2 samples could be in consequence of the high level nutrients found in the medium, which does not appear to counteract with the antimicrobial activity of honey. Therefore, *B. cereus* at that high concentration of 1×10^6 CFU/mL develops further in the more concentrated sample. Some authors have also reported *B. cereus* to be disaffected by honey antimicrobial activity. P.J. Taormina *et al.* (2001) undertook a study to compare U.S.A honeys from six floral sources for their inhibitory activity against *B. cereus* among other microorganisms. The disc assay revealed that development of zones of inhibition of growth depends on the type and concentration of

honey, as well as the test pathogen, being the growth of *B. cereus* the least affected. C. Voidarou *et al.* (2011) performed a bioassay for determining the antimicrobial effect on *B. cereus* (among others) by the well-agar diffusion method. The authors found *B. cereus* to be more resistant to honey antimicrobial activity.

However, such behaviour of *B. cereus* on rosemary honey does not agree to that found with most of the other authors, who have found the genus *Bacillus* to be inhibited by honey (G.F. Townsend, 1976; P. Molan,1992; J. Snowdon and D. O Cliver,1996). For instance, the antimicrobial activity of 10 honey samples from south Riyadh (Saudi Arabia) were investigated for their antimicrobial activities by using the agar-well diffusion method, against five standard microorganisms *S. aureus, B. subtilis, P. aeruginosa, E.coli and C. albicans* (T.H. Ayaad *et al.,*2011). The assay was done with three concentrations 30% (w/v), 70% (w/v) and undiluted honey. The authors reported all the tested strains were inhibited. Honey has been reported to inhibit the growth of lots of bacteria such as the microorganisms tested in this work (*Bacillus cereus, E.coli, Candida albicans, Staphylococcus aureus, Salmonella dublin and Shigella dysenteriae)* by S.N. El-Sukhon *et al.* (1994) and N. Ceyhan and A. Ugar (2001). P. Molan (1992) in his literature review reported *Bacillus cereus* to be inhibited at a concentration of 50 % (w/v) by agar diffusion assay.

However, it is difficult to compare the findings since the microbial load (CFU/mL) was not specified in any study and the type of honey used was different than the present study.
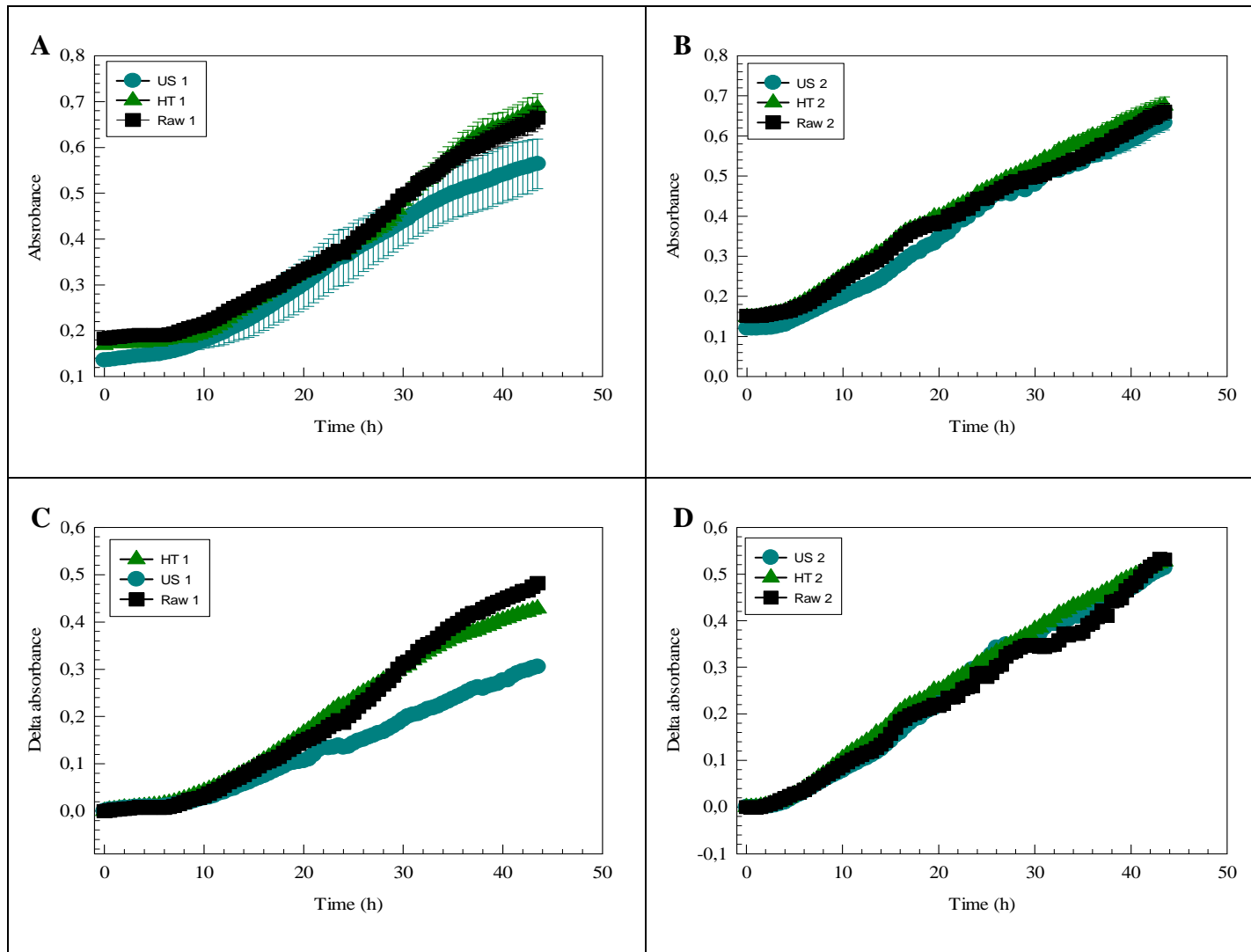
Figure 17. (A) *Bacillus cereus* 10^6 CFU/mL in (●) US honey (▲) HT honey, and (■) raw honey at a concentration of H1 (1g/2mL). **(B)** *B. cereus* 10^6 CFU/mL in honey H1 normalized growth curve. **(C)** *B. cereus* 10^6 CFU/mL in honey H2 (1g/1mL). **(D)** *B. cereus* 10^6 CFU/mL in honey H2 normalized growth curve.

Microbial concentration of 10³ CFU/mL

Figure 18(A) shows the microbial growth of *B. cereus* (1×10^3 CFU/mL) in presence of US, HT and raw honey sample H1 (1g/2mL). As the graph shows, the US-treated sample presented the initial lowest value among the samples, whereas the HT and raw honey samples started at practically the same point. Moreover, the curve of *B. cereus* growth in the US sample remained relatively flat throughout the incubation period (48 h) and the raise was only observed after 37 h of incubation. This is probably because honey has lost its activity by that time. However, it is important to note that *B. cereus*'s incubation time ranges from 5-16 h. Thus it can be assumed that the inhibition of *Bacillus* was achieved, and the growth observed later on, could be due to remaining spores that developed once the honey had lost all its activity. *B. cereus* in the HT sample developed gradually (practically at the same extent that raw honey sample) as it is observed a continuous and moderately rise in the absorbance curve within the first 20 h. *B. cereus* in presence of raw honey sample augmented from the start since it is perceived a slight but progressive increase in absorbance, and the rise became sharper after 28 h approximately until the end of the incubation. Figure 18(C) shows the normalized curve of *B. cereus* (1×10^3 CFU/mL) in US, HT and raw honey sample H1 (1g/2mL). Starting all samples at point 0, it is observed that the sonicated sample showed the least increase until 37 h of incubation, from which it presented a more pronounced increase. HT sample however, exhibited from the very beginning a slight increase throughout the experiment, and only when the sonicated sample started its increase it reached the absorbance of the HT sample over passing it slightly. Raw honey, on the contrary, did not show inhibition effect at all, since the increase of absorbance started from the commence and a sharp ascension was observed after 28 h of incubation. This graph (Fig.18) shows that the US treatment inhibits the microbial growth delaying its development, and thus increasing the shelf life of honey. As mentioned before, the incubation time of *B. cereus* takes place within 16 h at most, and therefore the inhibition effect should be observed in this time frame. In such wise, it is observed that the US treatment inhibited *B. cereus* the most, while the only HT treatment did not seem to inhibit *Bacillus*'s growth since it has the same absorbance value than raw honey sample.

Figure 18(B) shows the microbial growth of *B. cereus* (1×10^3 CFU/mL) in presence of US, HT and raw honey sample H2 (1g/1mL). The graph shows once again that the US treated sample started with the littlest absorbance point, whereas the HT and raw honey samples started with nearly the same absorbance. However, all samples exhibited a noticeably ascension after 16 h of incubation, indicating that *B. cereus* was not affected by any of the honey samples. However, the ascension was belittle in the US and HT sample at the end of the experiment, indicating a piddling inhibition effect. Figure 18(D) shows the normalized curve of *B. cereus* (1×10^3

CFU/mL) in US, HT and raw honey H2 (1g/1mL), which confirm the previous impression. Recurrently, this lack of inhibition when honey is more concentrated could be due to the variety and high nutrient inputs in the well, which support the microbial growth. The lack of inhibition of *B. cereus* may reflect a generally higher tolerance of *Bacillus* species to antimicrobials that may be present in honey. Moreover, gram-positive bacteria are in general more resistant to the effect of ultrasound than gram-negative bacteria (P. A. R . Vandamme, 2007; T. Monsen *et al.,* 2009). This difference is probably related to the fact that gram-positives possess a thicker and more robust cell wall due to cross-linking of peptidoglycan and teichoic acid, which make these bacteria less susceptible to ultrasound. On the other hand, glucose-oxidase activity (and therefore hydrogen peroxide production) varies substantially in different honeys, with some honeys showing no activity at all (L. Chepulis, 2008). This variation within honeys can be due to several reasons. The low levels of glucose-oxidase activity seen in some honey sample can be a result of the loss of activity that occurs during the processing and handling of the honey by the apiarists. The glucose-oxidase enzyme that generates the hydrogen peroxide is destroyed by excessive exposure to heat and light, so careful packaging and storage is required if this activity is to be preserved. Some authors have found that heating honey causes loss of activity against some species whilst it is retained against others (P. Molan, 1992). However, since raw honey sample did not exhibit any inhibitory effect on *B. cereus*, the possibility that the heat or ultrasonic treatment has affected the activity of glucose-oxidase can be discarded. In addition, honey was active to the rest of microorganisms, hindering their development. The level of hydrogen peroxide production is also affected by the floral source that the honey is made from. Many of the dark coloured honeys (i.e. blueberry, buckwheat) have been shown to have higher levels of hydrogen peroxide-induced bacteria-killing activity, whereas light coloured clover honeys were consistently found to have low activity (L. Chepulis, 2008).

So far, among the test pathogens, growth of B. cereus was the least affected by honeys.
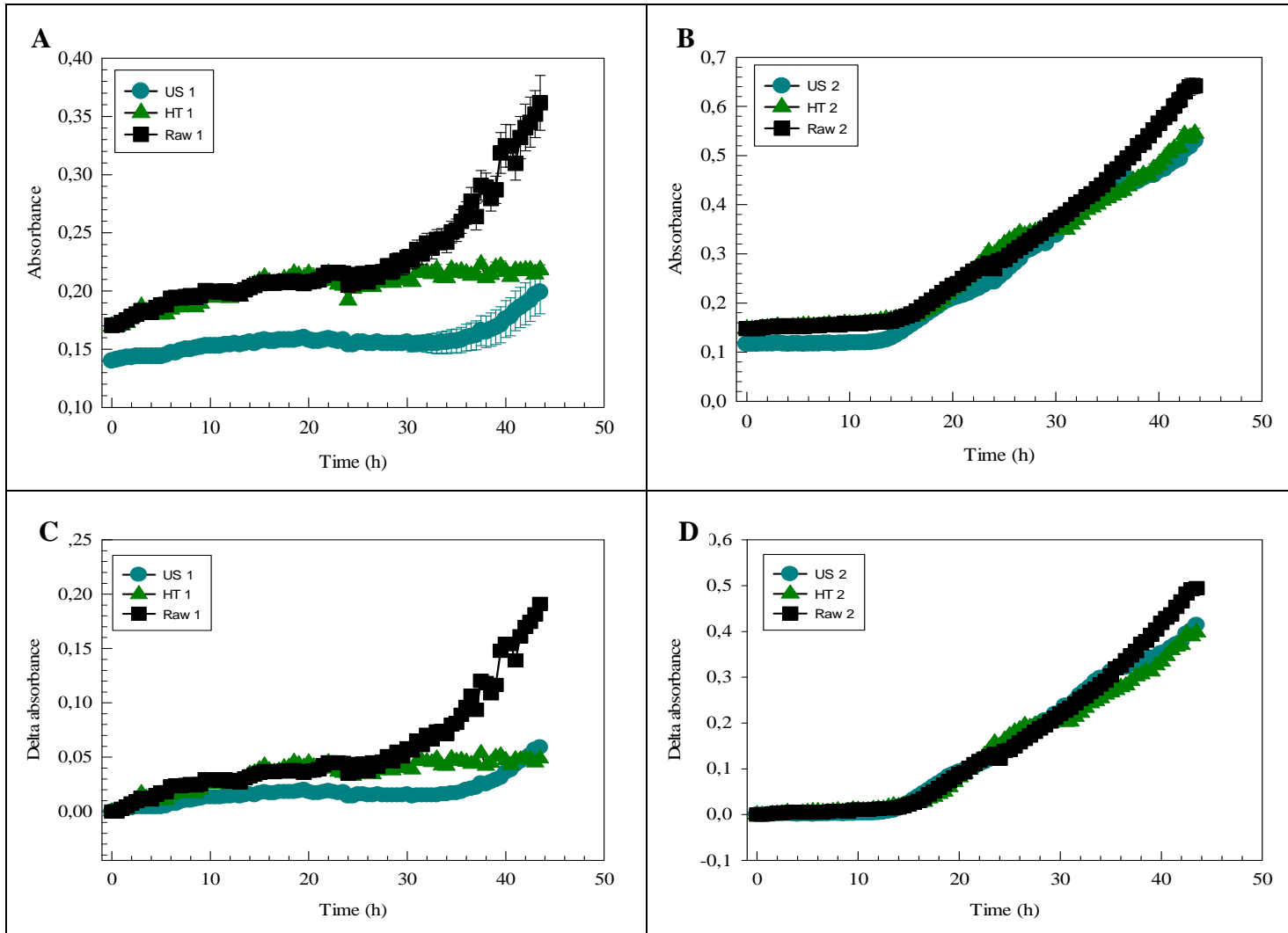
Figure 18.(**A**) *Bacillus cereus* 10^3 **CFU/mL in** (●) **US honey** (▲) **HT honey, and** (■) **raw honey at a concentration of H1 (1g/2mL). (B)** *B. cereus* 10^3 **CFU/mL in honey H1 normalized growth curve. (C)** *B. cereus* 10^3 **CFU/mL in honey H2 (1g/1mL). (D)** *B. cereus* 10^3 **CFU/mL in honey H2 normalized growth curve.**

Furthermore, with each plate analysis, 200 microlitres of diluted (1g /1mL and 1g /2mL) honey samples (raw, US and HT samples) were placed alone in the well-plate and incubated at 35 ± 2 °C. Then, the absorbance was measured for 24 h. With this experiment it was sought to observe the intrinsic antibacterial activity of the treated honeys and to confirm spectrophotometrically the results found in the count plate agar by reading the absorbance. Hence, it was presumed to observe a denoted increase in absorbance for raw and heat-treated samples, and a tinier increase in the sonicated samples.

Figure 19 shows the evolution of raw, sonicated (US) and heat-tread (HT) samples. As it can be seen, the US samples showed notable lower absorbance than those HT or the untreated (raw honey) ones, for both honey concentrations.

Figure 19(A) shows the diluted honeys curves of 1g/2mL, as it can be observed; the US honey presented almost a flat tendency indicating no microbial growth. The HT sample presented a clear rise of absorbance, which indicates microbial growth; however, when compared to the raw honey sample, the heat treatment also reveals microbial inhibition to some extent. Raw honey sample, on the other hand, exhibited a progressive absorbance increase which became sharper after 17 h of incubation, showing microbial growth and development.

Figure 19(C) shows the normalized curve of diluted honeys of 1g/2mL, it can be seen that the US honey presented the least increase, being essentially flat throughout the incubation time. Heat-treated sample showed a soft but gradual enlargement in absorbance, whereas the raw honey sample showed the sharpest increase of absorbance. These findings confirm the results observed in the agar plates (section 6.3.1.).

Figure 19 (B) shows the diluted honeys (1g/1mL) curves. As this honey sample is more concentrated, there was a greater increase in absorbance at the started point. This could be because the nutrients present in the well favours the remaining microorganism to develop. Nevertheless, the sonicated sample showed again the littlest increase whereas the HT and raw honey samples presented larger increases. The HT sample curve greatly developed but it was below the raw honey sample, from which it is appreciate a scanty effect of inhibition. Figure 19(D) represents the normalized curve of diluted honeys of 1g/1mL, which confirms the aforementioned discussion.
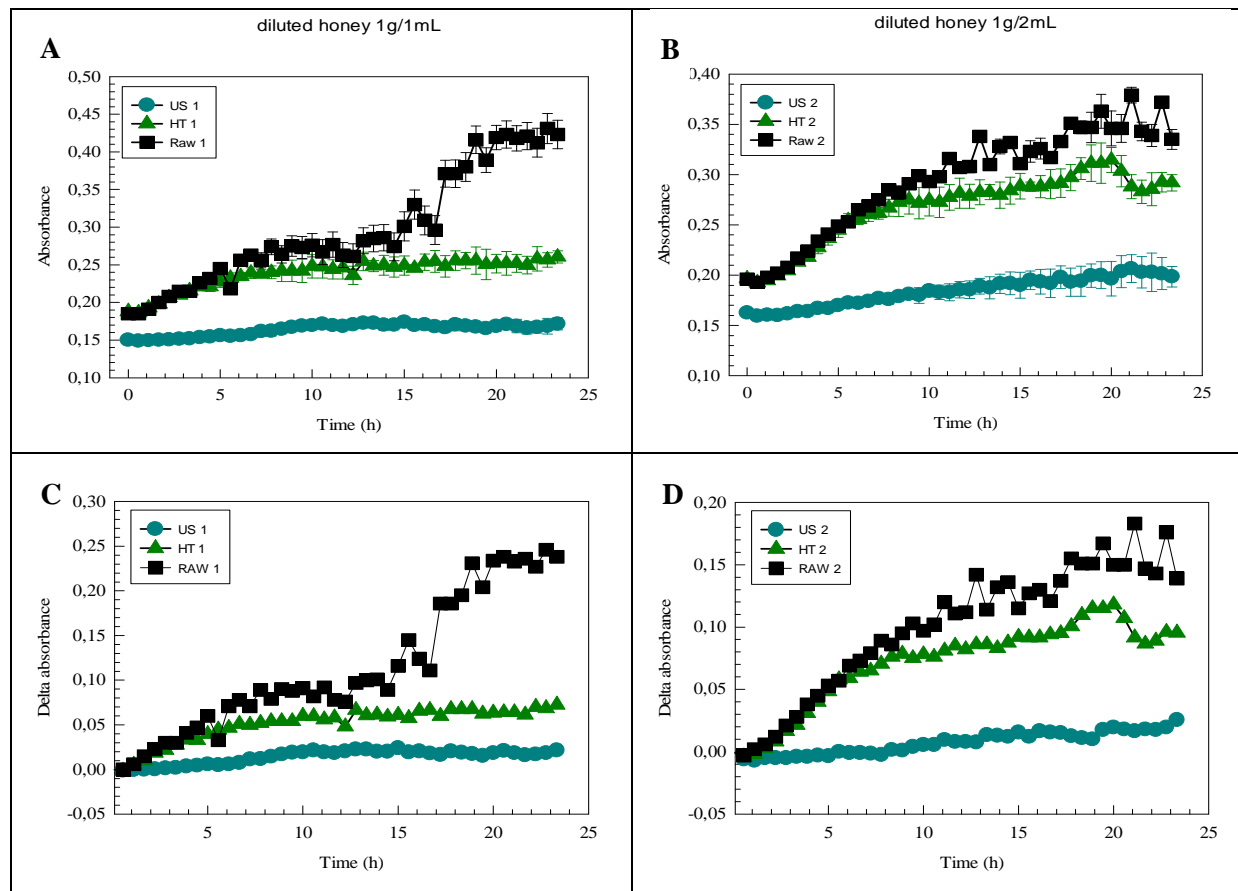
Figure 19. Diluted honey samples (●) US honey (▲) HT honey (■) raw honey incubated at 35 ± 2 °C for 24 h. (A) Honey concentration 1g/2mL (H1). (B) Honey concentration 1g/2mL (H2). (C) Normalized curve H1. (D) Normalized curve H2.

6.3.3. Determination of Hidroxymethylfurfural (HMF) content in honey by HPLC

In Chapter 2 honey samples were analyzed to determine whether an increase of HMF occurred after an ultrasound treatment. However, since in this Chapter the test conditions used to decontaminate the honey were different from the samples previously analyzed, the HMF content was evaluated again in samples treated at 50 °C for 2h. There were made 6 replicates for each sample; the only heat-treated and the sonicated ones.

All treated samples, however, did not exceed 1g of HMF /kg honey after the treatment at 50 °C for 2h. Ultrasound treated samples presented a concentration of 0.85 ± 0.01 mg/kg and the only heat-treated samples 0.83 ± 0.01 mg/kg. These findings agree with I. Turhan *et al.,* (2007) who also reported that honey did not contain significant amounts of HMF and neither increase at practicable mild temperatures.

Moreover, samples were analyzed again after 6 months of their treatment, to determine whether the HMF content was increasing over time. It is important to remark that all samples presented only a slight increase of HMF content; the US sample presented 1.12 ± 0.01 mg/kg and the heat-treated sample 1.09 ± 0.01 mg/kg. These findings indicate that fresh rosemary honey is not damaged by heat or ultrasound treatment and also lasts over time without being influenced its quality.

6.3.4. Conclusions

The present Chapter sought evaluating the effect of different combinations of ultrasound treatment (power, temperature and duration) on honey microbiological decontamination, and on the other hand, to assess whether the intrinsic biological activity of honey was affected after an ultrasound treatment.

1. The analyzed rosemary honey contained yeast and fungi much below 1×10^2 CFU/g, the maximum amount allowed by Codex Standard for Honey (Codex Stan 12-1981). The total bacteria (1×10^2 CFU/g) count was also far below the maximum limit value ruled (1×10^3 CFU/g).

2. Temperature treatments close to the optimal microbial temperature growth (37 °C) and short periods of sonication (20, 40 or 60 min) do not inhibit but promote the microbial expansion, as it acts as a stimulant. Both, the sonicated and only heat-treated samples, exhibited and increase in size and population when treated at 40 °C for 20, 40 and 60 minutes. It was found that the ultrasound treatment enhanced the microbial population (113 CFU/g) when compared to raw honey (87 CFU/g) and the conventional heat treatment (110 CFU/g). The yeast count was also higher.

3. The longer the treatment time the bigger the colonies. Samples sonicated for 60 min at 40 °C presented higher counts than those treated for 40 min, and likewise samples sonicated for 40 min presented higher and bigger colonies than those treated for 20 min.

4. Honey samples sonicated at 50 °C for 2 h inhibits considerably the size and microbial load, and delays the microbial growth by increasing its stationary phase.

5. The ultrasound treatment inhibited greatly the growth of *E.coli*. Honey samples at a concentration of 1g/2mL or 1g/1mL inhibited the growth of *E.coli* 1×10^6 CFU/mL up to half of the raw honey sample. At lower concentration of *E.coli* 1×10^3 CFU/mL, the ultrasound treatment delayed the lag phase but did not inhibit the growth.

6. *Candida amapae* and *Saccharomyces cerevisiae* at a microbial load of 1×10^3 CFU/mL and 1×10^6 CFU/mL were greatly inhibited by the sonicated honey sample at both concentrations of 1g/2mL and 1g/1mL. Heat-treated samples did

present inhibition but to a lesser extent, whereas raw honey did not show an inhibition effect.

7. *Bacillus cereus* at both microbial loads of 1×10^3 CFU/mL and 1×10^6 CFU/mL was barely affected by the honey samples. However, the highest inhibition effect was observed in the sonicated sample at a concentration of 1g/2mL. The more concentrated sample of 1g/1mL did not affect the growth of *Bacillus cereus* at none of the samples.

8. The US treated sample started with the minimum absorbance point, followed by the HT sample, whereas the raw honey sample started always with the highest absorbance, which means that US treatment liquefies better the honey.

7. General Discussion

Based on the experiments performed along this thesis, different combinations of ultrasound treatment (temperature and duration) can be used to achieve the main objective of the present study of honey deliquescence.

Several approaches were undertaken to evaluate the effectiveness of applying ultrasound treatment to liquefy naturally crystallised or candied honey.

Firstly, **in Chapter 1**, the effect of different combinations of ultrasound treatment (power, temperature and duration) on honey liquefaction were evaluated by studying the rheological properties of honey; viscosity behaviour, crystal content, tendency to re-crystallization and thermal properties.

Secondly**, in Chapter 2**, the effects of ultrasound on the hydroxymethylfurfural concentration and diastase activities in honey were determined by chemical analysis and compared with that for standard heat-treated honey samples.

Thirdly, **in Chapter 3**, ultrasound treatment was also investigated for honey decontamination. In addition, the *in vitro* antimicrobial and antifungal activities of ultrasonicated honey against several types of microorganisms were evaluated.

We have demonstrate that ultrasound treatment can be effectively used for thermal processing of honey, as it has been shown to speed up the liquefaction of honey. Moreover; it delayed the re-crystallization of honey, increasing its shelf life (Chapter 1). Furthermore, it has been demonstrated that the main quality parameters of HMF and diastase activity were not influenced by the ultrasound treatment and either was the sugar composition (Chapter 2). The ultrasound treatment was also investigated for honey decontamination (Chapter 3) and it was shown to inhibit considerably the size and microbial load. On the other hand, the intrinsic antimicrobial activity of honey was enhanced with ultrasounds.

Honey crystallization

Honey crystallization or granulation is a natural phenomenon by which honey turns from liquid (runny) state to a semi-solid state. This natural phenomenon happens when glucose, one of three main sugars in honey, spontaneously precipitates out of the supersaturated honey solution. Glucose tends to precipitate and the solution changes to the more stable saturated state. Some honeys crystallize uniformly; some will be partially crystallized and form two layers, with the crystallized layer on the bottom of the jar and a liquid on top (see Picture 23). Honeys also vary in the size of the crystals formed. Some form fine crystals and others large, gritty ones.

During crystallization water is freed and both crystalline and liquid phases may coexist during certain time. Consequently, water activity becomes greater than that of the original liquid honey. This is produced by a decrease in the solution concentration due to the release of water from the solid phase as a consequence of crystal formation. This makes the substrate conditions suitable to allow the development of the normally present microbial flora and, within time, to produce subsequent organoleptic modifications and quality damages. Thus, partially crystallized honey may present preservation problems. Crystallization is another important characteristic for honey marketing; partially crystallized or re-liquefied honey is not an attractive presentation for retail shelves (Picture 23) (NHB[a], n.d).

Picture 23. Honey solidifying and crystallizing. (Courtesy: F. Intoppa)

The time it will take the honey to crystallize depends mostly on the ratio of fructose to glucose. It has been observed that honeys with a high percentage of fructose remain liquid for a long time (NHB[c], n.d). Hence, honey high in glucose sugar, with a low fructose to glucose ratio will crystallize more rapidly, such as alfalfa, cotton, dandelion, mesquite, mustard and rape. Honey with a higher fructose to glucose ratio (containing less than 30% glucose) crystallizes quite slowly and can stay liquid for several years without special treatment, for example, robinia (black locust), sage, longan and tupelo. (K. Flottum, 2009). Rosemary honey analyzed in the present work presented an average concentration of 35% fructose and 31% of glucose which agreed with other authors (C. Pérez-Arquillué *et al.*, 1995; R. Mateo and F. Bosch-Reig, 1996).Therefore, rosemary honey in question is commonly classified as half prone to crystallization.

Honey liquefaction

As described in the introduction, the main goal of this thesis is to study the incidence of an ultrasound treatment on honey liquefaction. To that end, honey was treated in an ultrasound bath filled with distilled water.

Honey is heat processed at different temperatures and times around the world, or even different conditions are used within a country. In this manner, in Russia honey is heated at 57 °C for 1h, at 60 °C for 32 min, at 70 °C for 10 min or even at 80 °C for 2-4 min depending on the region (L. R.Verma, 1992). In Australia, honey is heated at 55 °C for 16h followed by heating at 72 °C for 2 min prior to packaging (B. d'Arcy, 2007). In North and South America, Italy and Turkey honey processors reach temperatures above the 65 °C throughout the processing line (G.F. Townsend, 1976; I. Turhan *et al.*, 2008) whereas in France honey is treated at 78 °C for 6-8 min (M. Gonnet, 1979). In Spain however, a gentler heating process is applied in order to slowly begin to liquefy the honey, usually heated at 40-45 °C for 48 h.

Hence, the temperature and time parameters considered more appropriate to use and therefore were chosen in this study were heating the honey at 40, 50 and 60 °C for 20, 40 and 60 minutes. It should be taken into account that it is a combined treatment of soft heating and ultrasounds. To evaluate its effectiveness it was compared to the conventional heat treatment (HT) at the same conditions.

First, the viscosity behaviour of ultrasonicated honey (US) was investigated (Chapter 1). Rosemary honey was found to follow a Newtonian behaviour. This fact agreed with other authors who reported that light-coloured honeys (like rosemary) follow a Newtonian behaviour (J. Abu-Jdayil *et al.*, 2002; P. A. Sopade *et al.*, 2003; L. Juszczak and T. Fortuna, 2006; S. Yanniotis *et al.*, 2006).

When treated, honey became liquid. However, there was a clear difference in the viscosity decrease between the sonicated samples and the only heat treated samples. The action of the ultrasound treatment liquefied the honey in a shorter time interval than the conventional treatment. The findings obtained in this work agree with those reported by other authors. T. Kalogereas (1955) reported that high frequency sound waves (9 kHz) eliminated the existing crystals and retarded further crystallization. Later, Liebl (1977) decreased the time of honey liquefaction to 30 s using ultrasonic waves at a higher frequency (18 kHz) in a temperature range of 10-37 °C. The author reported 1500 kg of honey were liquefied in1 h, which reduced the cost significantly. However, his work does not mention the quality parameters of honey after being sonicated. In the work of A. Thrasyvoulou *et al.* (1994) crystallized honey (100 g) was liquefied by ultrasonic waves at 23 kHz in an interval period time of 18-25 minutes. Nevertheless, the authors stated that the temperature treatment increased up to 80 °C and thus the honey quality was degraded. Last, B. d'Arcy (2007) liquefied creamed honey by

introducing a sonotrode inside a vessel (200 ml) full of honey. The temperature reached ranged from 66.2 to 67.8 °C in an interval time of 5.4 – 6.3 min. The author stated that the mechanical disintegration of crystals during ultrasound treatment was due to cavitation.

The size of cavitation bubbles generated by an ultrasound sonotrode processor inside a glass vessel can be measured by a laser diffraction instrument, the Malvern Mastersizer 2600 (F. Burdin *et al.*, 1999). Late, to better understand the effect of ultrasound on honey, B. d'Arcy (2007) carried out a study of cavitation (bubble formation) produced by ultrasound in pure water and sugar solutions. However, there were difficulties in locating the glass vessel, filled with distilled water or fructose solution, in the Malvern Mastersizer/E and aligning the optical measuring unit to obtain the required laser intensity.

The tests for the 20 %, 30 % and 40 % fructose solutions were undertaken satisfactorily. However, it was not possible to obtain satisfactory laser intensity data for the 60 % and 80 % fructose solutions using the Malvern Mastersizer/E. The results for the 20 % to 40 % fructose solutions indicate that values for bubble diameters were in the range of 0 – 600 μm. However, for the more concentrated sugar solutions, the plot of particle size *versus* percentage frequency by volume, and the plot of particle size *versus* percentage undersize by volume, were incomplete and the measurements were limited at a maximum to 600 μm. It was concluded that this method was not able to be used to measure cavitation yield since the Malvern Mastersizer/E was unable to capture the bubble field with sufficient laser intensity, and that cavitation study was abandoned.

Second, to further study the crystallization honey process, ultrasound treatment was investigated as a means of reducing the crystal size, and thus improving the spreadability of the product (Chapter 1). Samples obtained from each of the ultrasound-treated honeys were examined under an optical microscope to observe the dissolution of crystals and the incorporation of air bubbles. In addition, visual observations on the status of honey in the containers were carried out after each treatment condition (Chapter 1 point 1.3.2.). Prior to the start of this project it was thought that ultrasound treatment of the D-glucose monohydrate crystals in honey would shatter them, thereby reducing their size. However, B. d'Arcy (2007) reported in his work that rather than reducing the crystal size through breakup of the crystals, the crystal size is reduced through partial melting or dissolution of the D-glucose monohydrate crystals. The images obtained in the present work are consistent with his findings. The clean, sharp crystals structures (plates) were replaced by irregular shaped plates, indicating that some glucose molecules on the edge of the crystal structure dissolved into the surrounding liquid, producing plate crystals that have melted edges and surfaces (see Pictures 8, 9, 10 and 11).

To complete the ultrasound liquefaction study, it was necessary to determine the effect of ultrasound treatment, relative to heat treatment, on the stability of liquid honey with respect to

subsequent crystallisation. It is a common problem within the honey industry for heat-treated liquefied honey to crystallise on storage, particularly during cold weather. Since liquid honey is preferred by most consumers, and by food companies (for ease of handling), then a method to retard the crystallisation process in honey is required.

So third, the storage behaviour after the ultrasound treatment was evaluated for long-term storage (Chapter 1). The tendency of re-crystallization was evaluated for both sonicated and heat-treated honey samples. More needle crystal masses were formed in the heat-treated honeys than were produced in the ultrasound-treated honeys at the end of the monitoring period of 2 years (from 2009 to 2011). Ultrasound treatment delayed D-glucose monohydrate crystallisation more than it did a heat treatment similar to that used by the honey industry. This occurs at both the microscopic level (in a drop of honey) and in bulk samples (Picture 13). These results agree with those found by B. d'Arcy (2007) who also studied the re-crystallization phenomenon of Australian honey. The author also reported to be a difference in the crystal formation process at the microscopic level in ultrasound-treated honey relative to that in heat-treated honey.

Fifth, the honey thermal properties of glass transition temperature and change of enthalpy of US honey were studied using a DSC (Chapter 1).

The inflection Tg of Raw, US and HT honey samples were observed at -42.03 °C, -42,70 °C and -41.68 °C, respectively, which was in good agreement with the literature values from -33.64 to -51.14 °C (J. Ahmed et al, 2007). The specific heat capacity (Cp) of the present study for raw honey was 2.72 J/ g °C, which agreed with other authors (2.26 J/ g °C, J.White (1975), 1.97-3.07 J/ g °C (P. A. Sopade *et al.*, 2005). The specific heat capacity of the US samples was 2.37 J/g °C and 2.62 J/g °C for the HT samples. These slightly differences indicate that honey is more sensitive to a temperature change (requires less energy) once treated, and that the combination of heat and ultrasounds falls more in the honey consistency.

On the other hand, raw honey presented the highest enthalpy of 251.72 J/g followed by the heat-treated (214.78 J/g) and the ultrasound-treated sample (211.92 J/g).Thus, the effect of ultrasounds on honey liquefaction (glucose crystal dissolution) is much incident than only a heat treatment.

Thus, ultrasound treatment will not only liquefy candied honey without the need for long exposure to high temperatures, but may make the liquefied honey more stable to subsequent crystallisation on storage.

Ultrasounds in honey quality

In Chapter 2 the two main quality indicators of hydroxymethylfurfural (HMF) and diastase activity (DN) as well as the sugar content of ultrasonicated rosemary honey were evaluated.

The diastase activity and the hydroxymethylfurfural (HMF) content are widely recognised parameters in evaluating the freshness of honey. International, European and Spanish legal regulations set a minimum value for diastase activity of eight on Gothe's scale, and a maximum HMF content of 40 mg/kg of honey (CODEX STAN 12-1981, 2001; EC Directive 2001/11, BOE 1049/2003).

Although in the last years many works have focused in the kinetics formation of HMF in honey (E. Tosi *et al.*, 2002; B. Fallico *et al.*, 2004; I. Turhan *et al.*, 2008), this compound is formed differently depending on the heat regime applied and the type (composition) of honey. F. Dimins *et al.* (2006) pointed out the formation of HMF at 60 °C; however, its formation is due to a combination of temperature and time. For example, heating the honey at 90 °C for 15 min results in a concentration of 0.80 ± 0.25 mg HMF/kg, while a concentration of 1.33 ± 0.11 mg/kg is found at 75 °C for 90 min (I. Turhan *et al.*, 2008). S. Ajlouni *et al.* (2010) found an increase of 8 mg/kg of HMF heating the honey at 85 °C for just 2 min.

In the present work, it was found that either sonicating or only heating at 40 °C, 50 °C or 60 °C for 60 min in rosemary honey type, the HMF content was still far away from the international limits (lower than 40 mg kg^{-1}). These results are in accordance with I. Turhan *et al.* (2008), which also did not found a significant HMF increase ($P > 0.05$) after heating the samples at 90 °C for up to 90 min in floral honeys, and up to 75 min in honeydew honeys.

In the present research, the initial amylase activity (raw honey sample) ranged from 9.35 to 10.34 DN. Although these values are relatively low compared with other honey types (e.g. Heather (Calluna, Erica sp.) DN of 22, they accord with other rosemary honey values of 3-10 DN (E. Mendes *et al.*, 1998; P. Oddo *et al.*, 1998).

P.Oddo *et al.* (2006) suggests that these lower values may be because the rosemary honey type is collected in early spring when the colony feeds brood and young bees, whose glands produce fewer enzymes. Even thought, heating honey samples in a water bath for 20, 40 and 60 minutes at 40 °C, 50 °C and 60 °C did not affect the level of amylase inactivation. As for the US treatments, no one of the different US-treated samples presented a lower DN value. The ANOVA test also corroborated there were no significant differences ($P > 0.05$). So, applying a 40 kHz frequency for the times mentioned above does not seem to affect the enzymatic activity.

Sugar composition plays an important role in determining the physical and chemical properties of honey. Fructose is the only carbohydrate that becomes HMF whereby the fructose to glucose ratio of honey affects the rate at which the HMF is generated. In the present research, rosemary honey presented an average fructose concentration of 36g/100g and 31g of glucose/100g of honey, which agreed with those concentrations found in rosemary honey by other authors (C. Pérez-Arquillué *et al.*, 1995). On one hand, being the ratio of

fructose/glucose 1.14 ± 0.01, a very low initial HMF concentration was expected. On the other hand, this ratio indicates this kind of honey has a mid tendency to crystallize.

The ratio fructose/glucose obtained in this research is almost in the range proposed by J. Pourtallier and Y. Taliercio (1970) for the rosemary honey (1.06-1.13). C. Pérez-Arquillué *et al.*, 1995 also obtained a ratio which is as well slightly out of the range (1.17 ± 0.01) for the rosemary honey type. In either case, no change in sugar composition was observed after only heating or applying ultrasounds in none of the treatment conditions.

Ultrasounds on honey decontamination

Microorganisms that survive in honey are those that withstand the concentrated sugar, acidity and other antimicrobial characters of honey (P.B. Olatain *et al.*, 2007). Primary sources of microbial contamination are likely to include pollen, the digestive tracts of honey bees, dust, air, earth, and nectar. Soil and flowers may be also sources of yeasts in honey. These sources are very difficult to control. Secondary sources are those arising from honey manipulation by people that include food handlers, cross-contamination, equipment and buildings (M. S. Finola *et al.*, 2007; NHB[a], n.d). Because bacteria do not replicate in honey, if high numbers of vegetative bacteria were to be detected, it may indicate contamination from a secondary source (NHB[a], n.d).

Indeed, medicinal importance of honey has been documented in the world's oldest medical literatures, and since the ancient times, it has been known to possess antimicrobial property as well as wound-healing activity. The mechanism of this function relates to its high osmolarity, acidity, and content of inhibines, such as hydrogen peroxide, flavonoids, and the phenolic acids (caffeic and ferulic acid). However, the antimicrobial ability of honey differs depending on its floral source (J.W. White, 1975; E. Crane, 1984). The antimicrobial agent in some honeys is predominantly hydrogen peroxide, and is formed by glucose oxidase synthesized by the bee. In other honeys, the antimicrobial agent is a non-peroxide compound derived directly from the flower. Hence, not every type of honey has equally effective antimicrobial properties. Is there therefore a contradiction in the medical significance of honey, which on one hand contains several microbes and on the other is active against many organisms?

Rosemary raw honey incubated in growth mediums presented microbial contamination (refer to Chapter 3 Picture 1). Nevertheless, these values were far below the maximum permissible statutory level (CODEX STAN 12-1981, 2001). Honey samples sonicated at 50 °C for 2 h inhibited considerably the size and microbial load, and delayed the microbial growth by increasing its stationary phase. This fact is important since the honey can be decontaminated without increasing the temperature treatment and thus not compromising its quality.

Honey antimicrobial activity

Raw, US and HT rosemary honey were tested against several microorganisms to study its intrinsic antimicrobial activity at two concentrations, 1g/1mL and 1g/2mL with a microbial load of 1×10^3 CFU/mL and 1×10^6 CFU/mL. Most authors tested the inhibitory effect of honey by agar well diffusion method and broth dilution method. In the present research however, the antimicrobial activity has been tested spectrophotometrically by broth microdilution method. All microorganisms studied in the present work were affected the most by the US treated honey. This could be because of the combined work of the US treatment and the antimicrobial characters of honey (i.e. glucose-oxidase activity) which activity is increased once diluted.

E.coli was found to be inhibited by sonicated honey at both concentrations and in a lesser degree by HT honey samples, while raw honey gave no indication of inhibition. These results agree with that found with other authors. In fact, the susceptibility of *E.coli* on honey has been widely reported by different authors around the world. As previously discussed in Chapter 3, H. Selçuk and K. Nevin (2002) tested the antimicrobial effects of different Turkish honey samples on gram-negative bacteria. Most of the honey samples analyzed were inhibitory at 50% and above concentrations on *E.coli*. P. Molan (1992) also reported *E.coli* to be inhibited by different types of honey. He even reviewed that *E.coli* is inhibited by artificial honey (38.4% fructose and 30.3% glucose dissolved in sterile water) when diluted 1 in 10 due to the osmotic effect. K. Brudzynski and L. Kim (2011) analysed Canadian honeys from diverse floral sources and were also screened against *Escherichia coli* (1×10^6 CFU/mL). Their results confirmed the inhibitory effect on *E.coli*. They also found that artificial honey (25% v/v) inhibited the microbial growth due to honey osmolarity. T.T. Adebolu (2005) evaluated the antimicrobial activity of two Nigerian honeys some bacterial species that cause diarrhea by agar well diffusion method. *E.coli* was found to be inhibited in a concentration of 1:8 of honey in sterile distilled water (v/v) for both honey samples. J. M. Wilkinson and H.M.A. Cavanagh (2005) determined the antibacterial activity of 13 Australian honeys, including 3 commercial ones, using standard well diffusion methods. All honeys tested had an inhibitory effect on the growth of *E.coli* with one honey still having activity against *E.coli* at 2.5%, but no honey was active at 1% concentrations. C. Basualdo *et al.* (2007) carried out a study to determine the antibacterial activity of 10 honeys produced in Argentina. However, the authors only found inhibitory effect in 4 of 10 of the undiluted honey samples, and no inhibitory effect in undiluted honey at 50 of 75%. D. P. Mohapatra *et al.* (2010) evaluated the *in vitro* antibacterial activity of methanol, ethanol, and ethyl acetate extracts of raw and processed honey against gram-positive and negative bacteria (1×10^8 CFU/mL). The authors reported that both types of honey showed antibacterial activity against the tested organisms. Gram-

negative bacteria were found to be more susceptible as compared to Gram-positive bacteria. Among all the extracts analyzed, the authors found the methanol extract to be the most effective as an antibacterial agent.

Bacillus cereus was also tested for honey antimicrobial activity. This microorganism showed great resistance to all the honey samples tested. As widely discussed in Chapter 3, the lack of inhibition of *B. cereus* may reflect a generally higher tolerance of *Bacillus* species to antimicrobials that may be present in honey. Moreover, gram-positive bacteria are in general more resistant to the effect of ultrasound than gram-negative bacteria (P. A. R. Vandame, 2007; T. Monsen *et al.*, 2009). This difference is probably related to the fact that gram-positives possess a thicker and more robust cell wall due to cross-linking of peptidoglycan and teichoic acid, which make these bacteria less susceptible to ultrasound.

Some authors have also reported *B. cereus* to be disaffected by honey antimicrobial activity. P.J. Taormina *et al.* (2011) undertook a study to compare U.S.A honeys from six floral sources for their inhibitory activity against *B. cereus* among other microorganisms. The disc assay revealed that development of zones of inhibition of growth depends on the type and concentration of honey, as well as the test pathogen, being the growth of *B. cereus* the least affected. C. Voidarou *et al.* (2011) performed a bioassay for determining the antimicrobial effect on *B. cereus* (among others) by the well-agar diffusion method. The authors found *B. cereus* to be more resistant to honey antimicrobial activity.

However, such behaviour of *B. cereus* on rosemary honey does not agree to that found with most of the other authors, who have found the genus *Bacillus* to be inhibited by honey (E.Crane, 1983; P. Molan, 1992; J. A. Snowdon and D. O Cliver, 1996). For instance, the antimicrobial activity of 10 honey samples from south Riyadh (Saudi Arabia) were investigated for their antimicrobial activities by using the agar-well diffusion method, against five standard microorganisms *S. aureus, B. subtilis, P. aeruginosa, E.coli and C. albicans* (T.H. Ayaad *et al.*, 2011). The assay was done with three concentrations 30% (w/v), 70% (w/v) and undiluted honey. The authors reported all the tested strains were inhibited. Many of the dark coloured honeys (i.e. blueberry, buckwheat) have been shown to have higher levels of hydrogen peroxide-induced bacteria-killing activity, whereas light coloured clover honeys were consistently found to have low activity (L. Chepulis, 2008).

On the other hand, two yeast where tested for antifungal activity of honey. *Candida amapae* was notably affected by the US honey when compared with the rest of the samples. A number of previous studies have demonstrated that various honeys, both commercially and locally produced have antibacterial and antifungal activity. P.E.Lubsy *et al.* (2005) performed an agar dilution method to assess the activity of Australian honeys against 13 bacteria and 1 yeast (*Candida albicans*). The honeys were tested at five concentrations ranging from 0.1 to 20% (v/v). Little or no antibacterial activity was seen at honey concentrations below 1%, with

minimal inhibition at 5%, however the greatest inhibition was seen at 20%. *C. albicans* was only inhibited at concentrations of 20%. In contrast of these authors, T. Ijaz *et al.* (2008) who also performed an agar dilution method reported the yeast *Candida albicans* was not inhibited by the Pakistani honeys at 20%. B.C. Nzeako and J. Hamdi (2000) in their study of six commercial Saudi-Arabian honeys found that honey inhibited *C. albicans* at honey concentrations higher than 40%, although the zone of inhibition was small compared with other organisms. A.C. Koc *et al.* (2009) evaluated the effect of Turkish honey samples from different floral sources for their ability to inhibit the growth of 40 yeast strains (*Candida albicans, C. krusei, C. glabrata and Trichosoporon spp.*). Broth microdilution method was used to assess the activity of the honeys against yeasts at different concentrations ranging from 1.25-80 % (v/v). All of the yeast strains tested were inhibited by honeys in their study. The study of M.L.Estevinho *et al.* (2011) assessed the in vitro antifungal properties of Portuguese lavender honey (10-60 % (v/v)) against *Candida albicans, Candida krusei, and Cryptococcus neoformans*. The results showed that the increase of lavender honey concentrations caused a decreased in the specific growth rate of all organisms studied. M. Kuçut *et al.* (2007) performed a study designed to assess the in vitro biological activities of three Turkish honey samples (Chestnut, Heterofloral and Rhododendron). A simple susceptibility screening test was employed against *C. albicans* and *C. tropicalis* (1×10^6 CFU/mL) among others microorganisms. Antimicrobial activities of the honeys were tested by using methanolic extracts of 50% concentration. Two honeys presented moderate inhibition against *Candida* species whereas one type did not show inhibition effect at all (Rhododendron).

Saccharomyces cerevisiae was the other yeast tested for honey antifungal activity. In contrast with other authors, *S. cerevisiae* was evidently inhibited by the honey. Although so far there are now only few works published testing the antifungal activity of honey against *S. cerevisiae,* the authors reported a lack of inhibitory effect against *S. cerevisiae.* A. Laorpaksa *et al.* (1992) evaluated the antifungal activity of 10 types of Thai honey on *Candida albicans* and *Saccharomyces cerevisiae*. The authors reported that none of the honey tested presented inhibition effect on these two microorganisms despite the concentration (10-50 %) used. S. Gomes *et al.* (2010) evaluated the antimicrobial effect of Portuguese honey against fermentative yeasts. Honey samples were pasteurized at 75 °C for 30 min prior to analysis. Yeast suspensions (approximately, 10^6 CFU/mL) were mixed in 0 %, 10 %, 25 % and 50 % of honey and made up to 25 mL with yeast broth. *S. cerevisiae* showed only a slight sensitivity to honey, being barely affected by the different honey concentrations tested. The authors suggested these results could be justified by the fact that *S. cerevisiae* yeast has been isolated from honey, so the yeast is probably adapted to the stress conditions found in honey. In fact, there are many studies evaluating the capacity of *Saccharomyces cerevisiae* strains,

isolated from honey to produce mead, a traditional drink, which results from the alcoholic fermentation of diluted honey carried out by yeasts (M. Navratil *et al.,* 2011; A. P. Pereira *et al.*, 2009; A. Mendes-Ferreira *et al.* 2010).

It is noteworthy that honey concentrations vary greatly from one study to another. Part of the explanation for the diversity in concentrations may be due to methodological differences between studies, the type (composition) of honey being used and the microorganisms tested. Usually, studies performed by agar diffusion use lower concentrations, while those of broth microdilution usually employ higher. This is probably because microorganisms in the liquid broth are more active because they have more mobility and there is more diffusion of nutrients, so they are more likely to develop rather than if they remain trapped in the agar. Moreover, the microbial number, as seen, also plays a crucial role in development.

In this study it has been used the broth microdilution method to assess spectrophotometrically antibacterial and antifungal activity rather than agar well diffusion, that is the most common method for testing honeys. In broth microdilution methods the honey is incorporated directly into the growth media; hence, the microorganisms are brought into direct contact with all honey components immediately on application to the well rather than relying on diffusion of constituents through the agar.

Several factors may influence the antifungal activity of honey. For example, J. H. DeMera and E. R. Angert (2004) reported that honey from different phytogeographical regions varied in their ability to inhibit the growth of yeasts, suggesting that botanical origin plays an important role in influencing the antifungal activity. In addition, there are a great variety of components, including phenolic acids, flavonoids and other biomolecules, in different honeys. Biological activity of honey is mainly attributed to the phenolic compounds (L. Estevinho *et al.* 2008).

8. General Conclusions

1. Different combinations of ultrasound treatment (temperature and duration) can be used to achieve the main objective of honey deliquescence. Ultrasound treatment can be effectively used for thermal processing of honey, as it speeds up the liquefaction of honey (i.e.9 min of US treatment *vs.* 23 min of only HT) in a temperature range of 40–50 °C. This indicates that honey can be liquefied by US waves without the need to increase the temperature up to 50 °C or even higher.

2. The analysis substantiated that US treated samples are prone to liquefy faster (i.e. 9 min of US treatment *vs.* 23 min of only HT) as the activation energy of the US treated sample was higher (20 kJ g^{-1} mol^{-1}) than the HT samples (16 kJ g^{-1} mol^{-1}), once treated at the same temperature. The E_a obtained from Arrhenius model of the US samples was lower (59 kJ g^{-1} mol^{-1}) which indicate that US-treated samples are the least sensitive (lower E_a value), where as HT samples are the most sensitive (highest E_a value of 64 kJ g^{-1} mol^{-1}) among the treatments examined.

3. The crystal content of ultrasound-treated samples presented a clearer and transparent honey than only heat-treated honey samples. The amount and size of the crystals were also the smallest.

4. Ultrasound-treated samples presented lower absorbance values in respect the only HT samples, which mean honey is more clear, limpid and translucent. Moreover the increase of absorbance over time (6 month period) was lower in the US samples. This fact indicates that ultrasounds liquefy better the honey and delays the re-crystallization than only a heat treatment, thereby having a longer shelf life.

5. For long-period storage (i.e. 2 years) at 4 °C, US samples took longer to re-crystallize, and at 50 °C no crystallization was observed in the US samples. These findings indicate that an ultrasound treatment at 50 °C is effective to hinder the crystallization and therefore it is not require a higher temperature treatment.

6. For the HMF concentration and the diastase activity, no detrimental effects were found from the ultrasound treatments relative to those observed from a normal heat treatment of honey.

7. Moreover, HMF formation in honey was fitted to a first-order reaction and activation energies (E_a) were calculated according to Arrhenius model. The E_a obtained of 31.06 kcal/mol, revealed that an US treatment did not overcome the energy barrier to carry out the reaction of HMF formation.

8. The sugar content in terms of fructose (36%) and glucose (31%) was not altered after sonication, and all ratios and sugar proportions remained constant after sonication. The fructose/glucose ratio of 1.14 ± 0.01 let associating the honey with an intermediate tendency of granulation.

9. The initial a_w value of the rosemary honey obtained was 0.613. Moreover, the a_w of the US-treated samples varied within the range 0.587–0.589 indicating that an US treatment could slightly decrease the a_w value and thus preventing the deterioration of honey by microbial contamination.

10. The analyzed rosemary honey contained yeast and fungi (4 CFU/g) much below 1×10^2 CFU/g, the maximum amount allowed by Codex Standard for Honey (Codex Stan 12-1981). The total bacteria (1×10^2 CFU/g) count was also far below the maximum limit value ruled (1×10^3 CFU/g).

11. Temperature treatments close to the optimal microbial temperature growth (37 °C) and short periods of sonication (20, 40 or 60 min) do not inhibit but promote the microbial expansion, as it acts as a stimulant.

12. Honey samples sonicated at 50 °C for 2 h inhibits considerably the size and microbial load, and delays the microbial growth by increasing its stationary phase.

13. The sensory analysis by the consumer panel revealed no preference between the US and HT samples. The only exception observed was the visual evaluation, where there was significant preference since 17 of 20 preferred the US sample, while 2 consumers preferred the HT and 1 consumer wrote down "no-preference". However, these results are only an approximation of consumer's preferences.

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