Katalin Sólyom

Enhanced extraction of natural substances using microwave energy

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Universidad de Valladolid

ESCUELA DE INGENIERÍAS INDUSTRIALES

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y TECNOLOGÍA DEL MEDIO AMBIENTE

TESIS DOCTORAL:

Enhanced extraction of natural substances using microwave energy

Presentada por Katalin Sólyom para optar al grado de
Doctor por la Universidad de Valladolid

Dirigida por:

Prof. Rafael B. Mato
Prof. María José Cocero Alonso

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Memoria para optar al grado de Doctor,

con **Mención Doctor Internacional**,

presentada por la Ingeniera Química:

Katalin Sólyom

Siendo los tutores en la **Universidad de Valladolid**:

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Valladolid, Septiembre de 2013

UNIVERSIDAD DE VALLADOLID

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La presente tesis doctoral queda registrada en el folio número _____ del correspondiente libro de registro número _____

Valladolid, a _____ de_____ de 2013

Fdo. El encargado del registro

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Certifican que:

KATALIN SÓLYOM ha realizado bajo su dirección el trabajo *"Enhanced extraction of natural substances using microwave energy"*, en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Valladolid, a _____ de_____ de 2013

Fdo. Rafael B. Mato Fdo. María José Cocero Alonso

Reunido el tribunal que ha juzgado la Tesis Doctoral titulada *"Enhanced extraction of natural substances using microwave energy"* presentada por la Ingeniera Química Katalin Sólyom y en cumplimiento con lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011 acuerda conceder por_____ la calificación de _____.

Valladolid, a _____ de_____ de 2013

PRESIDENTE SECRETARIO

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Abstract

Nowadays, in numerous industrial extraction processes, technologies that can save the time, space, energy and solvent are welcome in order to achieve production intensification. This is of particular importance in those cases where complex natural raw materials are involved, and the biological ***cell wall disrupti***on step is blamed for the ***slow kinetics*** and low throughput in the process.

Among several mechanical, chemical, physical or physicochemical treatments of natural raw materials, the possibility of ***using microwaves*** has emerged. Due to the electromagnetic irradiation of the material, rapid heating up and evaporation of intracellular water can be achieved, and the resulting pressure gradient may lead to cell wall damage. Thus, the intracellular compounds of interest become more accessible after an efficient pre-treatment, and faster kinetics can be achieved in the subsequent conventional process.

In this Doctoral Thesis, three different raw materials and processes were studied in order to explore the effects of microwave pre-treatment on extraction process kinetics.

Firstly, microorganisms were used, such as ***wastewater sludge,*** in order to intensify biogas production by liberating any intracellular material, which thus became accessible for anaerobic bacteria in the digestion process. Due to the large cell variety present in wastewater sludge, the specific study of the cell structure becomes difficult to elucidate, although the overall process kinetics can be upgraded, just as in a conventional thermal pre-treatment.

Secondly, unicellular ***microalgae*** were chosen as better candidates for this purpose. Also, lipid extraction for biodiesel production, and high added value algal pigment extraction are at the centre of attention in the field of continuously developing sustainable process engineering. The study on different microalgae and extraction techniques presented here showed that microwave pre-treatments have the potential to improve extraction kinetics, and also higher extraction yields may be obtained.

Finally, the abundant viticulture in the region of Castile and Leon suggested the study of ***wine making by-products*** (***grape marc***) in order to obtain valuable compounds (polyphenols) with antioxidant capacity, via solid-liquid extraction. Compared to the two former raw materials, grape marc represents higher-level cell organization, where different cell wall types may have diverse responses to pre-treatments. Microwave pre-treatments were studied both in the presence and in absence of an extracting solvent. Only solvent assisted microwave irradiation led to the successful intensification of kinetics in the performed comparative study. As a competing novel technique, the use of ultrasounds was also assessed through these studies, and

similar results were found when compared with microwave assisted extractions. Both pre-treatment techniques were able to overcome the long extraction times and large solvent stocks required in the industrial extraction process. The possible thermal degradation of these valuable compounds was considered.

Besides the study of these processes and the analysis of the effect of microwave irradiation on different raw materials, special importance has been devoted to the ***determination of the energy absorbed*** by the materials and solvents during the processes. The fraction of electromagnetic microwave energy absorbed by the sample is subsequently dissipated as heat. This heat can be described using an energy balance, which takes into account the *sensible heat*, by the temperature increment in the system, the *latent heat*, by solvent evaporation, and finally the *heat loss* to the environment. Unfortunately, results from published literature on the aforementioned topics are hard to compare with each other due to the lack of this data. Information about absorbed energy would facilitate the obtaining of comparable results in different experimental conditions, which are indispensable for scaling up.

Based on the results discussed in this dissertation, further research can be performed in order to develop an industrial scale microwave assisted extraction process of grape marc polyphenols. Furthermore, the deeper study of the microwave effect on different cell structures and the role of the solvent in the microwave assisted extraction seem to be interesting issues to follow in the future.

“If you have built castles in the air, your work need not to be lost; that is where they should be. Now, put the foundations under them.” /Henry David Thoureau/

Scope and Aims

SCOPE

In industrial processes, where natural raw materials are involved, the substance of interest may have ***intracellular*** localization. In order to facilitate the accessibility of the product in question, the natural cells have first to be damaged. This primary stage of the processes is often recognised as the ***rate limiting step,*** causing slow process kinetics, and therefore leading to long operation times, low recovery efficiencies or high operation costs.

Microwave irradiation can improve cell wall rupture of high moisture materials. Due to the rapid heating up and evaporation of intracellular water, the pressure gradient may lead to cell wall damage. Besides the thermal effect of microwave energy, there may be a non-thermal effect, which is highly controversial in literature: the alternating dielectric field may be able to force the polarized side chains of the cell wall macromolecules to break their hydrogen bonds, and thus alter their structure.

The microwave technology is widely used for analytical purposes due to the above-mentioned features, achieving faster and more economical procedures. However, the electric energy used to transfer microwaves into any sample could lead to high operation costs in industrial scale applications. For this reason, an ***effective and short microwave pre-treatment*** on the natural material may be convenient prior to the conventional processing. This additional stage would not replace the existing process, but may modify the cell structure in order to facilitate the conventional method in continuation, thus ***intensifying the conventional process kinetics***.

AIMS

The aim of this Doctoral Thesis was to study the effect of microwave pre-treatments on processes with microorganisms (*wastewater sludge and microalgae*) and plant cells (*grape marc*) to facilitate intracellular material liberation and thereby enhance the subsequent conventional process kinetics.

In order to attain the aforementioned goal, the following ***partial objectives*** have been raised:

- Study the effects of microwave pre-treatments in the presence of solvent and in solvent free systems.
- Assess the application of ultrasound assisted extraction as a competing novel process to microwaves.
- Evaluate concepts for up-scaling, which are necessary to study in laboratory experiments:
  - Determination of the energy density absorbed on laboratory scale.
  - Interaction between microwaves and raw material for further process modelling.
  - Study of the thermal degradation of the substances of interest in different experimental environments.

“The reading of all good books is like conversation with the finest men of past centuries.” /René Descartes/

Theoretical background

INTRODUCTION

This PhD thesis is a compliation of six chapters to satisfy the study of the established objectives. As an introduction to the field, a comprehensive theoretical background is presented here in order to get familiar with the subject, starting with the nature of the different **raw materials** that are of interest in intracellular material liberation, and the corresponding conventional processes.

Various raw material types were selected in order to study the effect of microwaves on cellular systems and final products: ***Wastewater sludge*** is composed of very different prokaryotic cells, and simple eukaryotes, with no or low cellular organization. The aim is to destroy the cell walls in order to liberate intracellular martial for biogas production, and no special attention is paid to the released compounds. The large cell variety makes the specific study of the irradiation on the cell structures difficult. However, from this perspective, ***microalgae*** are good candidates as unicellular systems.

The extraction of lipids or pigments from different species are of interest in this field (in particular, *Nannochloropsis gaditana* and *Scenedesmus almeriensis*). By contrast to wastewater sludge, in the case of microalgae the heat treatment may alter the final composition of the product, which would negatively affect the process, despite the enhanced kinetics. In the constantly changing and evolving field of microalgae processing, an established standard process, which could be used as a reference, was not found. Therefore, both solid-liquid (dry microalgae - hexane) and liquid-liquid (wet microalgae – hexane) extraction were studied in terms of extraction kinetics enhancement. Although promising results can be obtained with microalgae, the dependence on the infrequent raw material supply complicates long term work.

The abundant viticulture in the region of Castile and Leon suggests the study of wine making by-products for obtaining valuable compounds with antioxidant capacity, via solid-liquid extraction. Based on a patented polyphenol extraction process of ***grape marc*** (mixture of grape skin and seed) (*Moro Gonzalez 2010*), process kinetics intensification was studied applying microwave irradiation and ultrasounds as different pre-treatment processes to the extraction stage. Compared to the former raw materials, grape marc shows higher-level cell organization, where different cell wall types may have diverse response to the pre-treatments. Despite the complexity of the raw material, pre-treatments are performed to overcome the long extraction times and high solvent amounts, taking into account the possible thermal degradation of valuable compounds.

In the second part of this theoretical summary, the mentioned ***pre-treatment processes*** are discussed as possible alternative methods of cell wall disruption and process kinetics intensification. Basic knowledge of microwave heating and ultrasound is presented, whereas the subsequent Chapters of the Thesis focus on their application.

Finally, the ***calculation of the absorbed energy*** and ***equipment efficiency*** are presented on laboratory scale. In the published literature on these aforementioned research topics, there is a lack of information on the amount of absorbed energy in the pre-treatment process. This fact usually leads to results from different research groups, which are incomparable. This Thesis attaches special importance to the determination of the absorbed energy, as being the right way to obtain comparable results in different experimental conditions, which are indispensable for scaling up.

The theoretical background here presented focusses on the nature of the raw materials, and separately, on the microwave and ultrasound pre-treatment concepts. A specific summary of literature about the application of microwaves or ultrasounds on the referred raw materials are presented in the subsequent chapters.

1. RAW MATERIALS, PROCESSES AND PRODUCTS

1.1. Wastewater sludge and biogas production

Municipal wastewater treatment plants generate large amounts of excess sludge as a by-product of the complex physical, chemical and biological processes. Further treatment and disposal of this residue is responsible for up to 50% of total operating costs. In the case of land application and incineration, sludge conditions must meet the disposal acceptance regulations by reduced volume, improved character and reduced health problems. To this end, water content must be reduced and unstable organic material has to be transformed into more stable compounds.

In the first step of water purification (Figure 1), 50-60% of the suspended solids (SS) are removed together with 30-40% of the biological oxygen demand (BOD). The primary sludge settled here is 97-99% water and contains mainly high putrescible organic matter. In the second biological step, aerobic microorganisms are responsible for the remaining BOD and SS removal, besides the elimination of nitrogen and phosphate. The next stage is a secondary clarifier where the overflow liquid is discharged as effluent, and the bottom sludge (98-99% water content) is partially removed and partially recycled into the biological system.

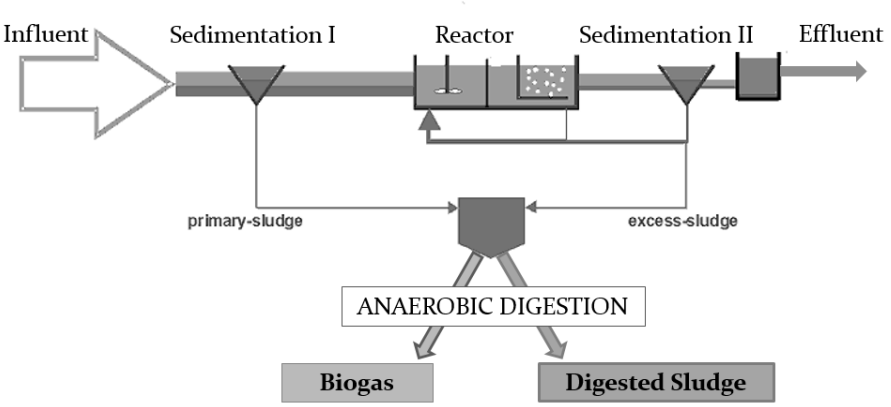

Figure 1 Schematic wastewater sludge treatment plant

The removed primary and secondary sludges are combined, thickened and further treated in various steps where anaerobic digestion is involved for biogas production. Thickening can be performed by gravity, flotation or belt filtration to decrease the sludge volume to one third of the initial value, and recycle the separated water to the wastewater treatment plant. In the anaerobic digestion, biochemical stabilization takes place: final solids are reduced, most of the pathogens are destroyed and organic matter is transformed into biogas, containing typically 60-70% of methane, besides CO_2 and H_2S. The obtained biogas can be a contributor to energy supply, having a heating value of 21-25 MJ/m^3, comparable to natural gas (37.3 MJ/m^3) (*Appels et al., 2006*).

Anaerobic digestion takes place as a sequential cooperative action of different bacterial strains when electron acceptors, such as sulphate or nitrate, are not available. In the first stage, facultative and obligate anaerobic bacteria release enzymes to facilitate the ***hydrolysis*** of initial proteins and polysaccharides to monomeric sugars, amino acids, long chain fatty acids and alcohols. The stage where insoluble organic material is degraded to soluble organic substances is considered as the ***rate limiting step*** of the digestion. The process is followed by the action of non-hydrolytic fermentative bacteria in the ***acidogenesis***, generating fermentation end-products such as acetate, formate, methanol, hydrogen and CO_2. In the next stage(***acetogenesis)*** obligate hydrogen producing acetogens facilitatesthe hydrogen transfer, while energy is gained by the bacterial growth on the compounds produced in the acidogenesis. The action of these bacteria is considered to be the connection between the initial fermentation stages and the final stage, ***methanogenesis***. In the last stage, strictly anaerobic *archea* strains are involved to produce methane from H_2/CO_2 reduction (*hydrogenophilic* sepcies) and by acetate carboxylation (*acetoclastic* species). However there are different pathways in methane production, 70% being produced via acetate carboxylation (*O' Flaherty et al., 2006*).

It is evident that excess sludge must be reduced through the wastewater treatment process, where it is produced. Different technologies have been studied so far in order to solve this problem, and enhance the process performance. Although reduction can be achieved either in the water line of the activated sludge process, or in the final waste line, by incineration or supercritical water oxidation, a wide range of techniques are available in the sludge line to reduce the final waste stream by enhanced anaerobic digestion. Physical, chemical or biological pre-treatments can be applied to sludge prior to the digestion step, while also modified digestion processes may also be performed in order to obtain better results. The disintegration of solid particles caused by pre-treatments leads to the release of intracellular compounds and facilitates the accessibility of substrates to anaerobic bacteria, thus accelerating the digestion process and increasing the degree of degradation. Although in the late '80s, mechanical cell disruption methods were preferred among physical technologies, nowadays the most successful treatments involve thermal hydrolysis, pressurized systems or alternative energy input by means of ultrasounds or microwave irradiation (*Chisti and Moo-Young 1986, Pérez-Elvira et al., 2006*).

The possible use of microwave energy as a sludge disintegration method prior to anaerobic digestion is deeply discussed in ***Chapter 1***. The influence of absorbed microwave energy and power is evaluated in terms of organic matter solubilisation and biogas production. In addition, a comparison with thermal treatment is performed to reveal a possible non-thermal microwave effect.

1.2. Microalgae: production of biodiesel and of high added value products

Microalgae are photosynthetic microorganisms that convert sunlight, water and CO_2 to algal biomass. This biomass is a good candidate in various scenarios, such as biofuels, foods, feeds and high added value compounds.

As renewable biofuels, methane can be produced by anaerobic digestion of algal biomass, while microalga oil is appropriate for biodiesel production, and biohydrogen can be photobiologically produced.

Biodiesel consists of triglycerides, where one glycerol molecule is esterified with three fatty acid chains. Through the transesterification with an excess amount of methanol in the presence of acid or alkaline catalyst, fatty acid methyl esters and glycerol are produced. For this process

to take place at atmospheric pressure at 60 °C, the necessary time period is 90 minutes. To avoid yield loss, both the oil and the reacting methanol must be dried to avoid saponification, and in addition free fatty acid content should not surpass a maximum value. In the final step, biodiesel is separated from glycerol and methanol by repeated washing with water (*Chisti, 2007*).

When considering the future replacement of fossil fuels, microalgae also have to be considered, as well as the amount of oil, produced from crops and animal fat at present. Microalgae might be an alternative solution due to the lower requirement in cultivation area and fast reproduction rates. Moreover, specific microalgae can provide extremely high oil yields for the purpose of biodiesel production (*Huang et al., 2010*).

The production of algal biomass is performed either in raceway ponds or in tubular reactors, where photosynthetic growth takes place under optimal conditions of CO_2, light, water and inorganic salt supply. The majority biomass produced in daylight must be withdrawn from the reactor, while the rest is consumed during the night via the respiration pathway. The processing of the harvested algae starts with a dehydration step (spray-drying, drum-drying, freeze-drying and sun-drying).

After the drying procedure, cells need to be disrupted in order to release the metabolites of interest (mechanical: cell homogenizers, bead mills, ultrasounds, autoclave, spray drying; and non-mechanical: freezing, organic solvent, osmotic shock, acid-base and enzyme reactions). Although it is difficult to achieve an energy efficient method in order to liberate the intracellular lipids from microalgae, it is necessary to facilitate and upgrade the extraction method, using a low solvent amount but still achieving high recovery of oil and minor high-value products. Several organic solvents can be used in the extraction process, such as hexane, ethanol or their mixtures, to recover up to 98% of purified fatty acids. The oil obtained is appropriate for transesterification in order to be converted into biodiesel, as has been described above (*Chisti, 2007, Mata et al., 2010, Scott et al., 2010*).

The replacement of fossil fuels has not been achieved so far by microalgae technology, since extensive research is needed to meet economical, environmental and consumer demand in the field. The process must be improved from an economic point of view at different stages. Metabolic and genetic engineering enhance algal biology to obtain high oil content strains, while advances in photobioreactor engineering can achieve more stable biomass production.

Finally, downstream processes (harvesting and oil extraction) have to be intensified in order to reach a sustainable biodiesel process (*Chisti, 2007, Huang et al., 2010*).

Besides the lipid content of microalgal cells, other valuable intracellular compounds are of interest in different industrial sectors such as fine chemicals and bioactive products. Pigments, antioxidants, β-carotenes, polysaccharides triglycerides, fatty acids, vitamins and biomass have applications with health benefits. Their importance is recognized and has been under extended research for the last thirty to forty years.

Sterols present in algae cells are used in cardiovascular disease prevention. Antioxidants and carotenoids (especially lutein) are important in the prevention and treatment of degenerative diseases, while algal polysaccharide complexes have immune-modulating properties. It is important to mention long-chain polyunsaturated fatty acids (PUFA), especially ω-3 and ω-6 types such as EPA, DHA or AA, which are pharmacologically important for dietetics and therapeutics. Those compounds are believed to have a positive effect on cardio-circulatory diseases, coronary heart disease, hypertension, cholesterol problems and in cancer treatments (*Mata et al., 2010*).

In the light of the great importance of substances with intracellular location in microalgae, ***Chapter 2*** was dedicated to the study of microwave energy application in order to enhance the extraction kinetics of both lipid and pigment compounds from different microalgae species.

1.3. Grape marc and antioxidant extraction processes

In the ***red wine making process*** the selected grapes, arrived from the field, suffer destemming and vigorous crushing in the first stage. No excessive crushing should be performed in order to avoid the increment of non-soluble solids and seed breakage, which leads to a high release of phenolic compounds. Depending on the wine type and grape quality, the fermentation conditions, temperature ramps and the contact time between the liquid and solid phases may vary to provide the desired taste, colour and phenolic component performance to the wine. During the process, sulphur dioxide is added to inhibit the polyphenol-oxidase activity and provide more stability to the phenolic components (*Zoecklein et al., 1995*). The discarded solid residue after the fermentation process, the so-called ***grape marc***, has a ***high content of bioactive compounds***. Taking into account the whole worldwide wine production, around ***9 million tonnes of residue*** is generated every year. Thus the demand for natural antioxidants to

replace synthetic additives in the food industry can be connected to the large amount of high added value product, recovered from the residue of the wine industry (*Lafka et al., 2007*). The recovery of active components is also a convenient way to achieve their removal from the grape pomace, so that it can be used as a fertilizer preventing inhibition problems (*Negro et al., 2003, Pinelo et al., 2006*).

Grape marc is a mixture of grape seeds, skin and a minimal amount of pulp. It contains active components such as flavonoids, polyphenols, anthocyanins, proanthocyanidins, procyanidins and stilbene derivatives. These components are of interest for the food-, cosmetics- and pharmaceutical industries due to their antioxidant, antimicrobial, antiviral or anticarcinogenic features (*Nassiri Asl and Hosseinzadeh 2009*).

Different types of polyphenols may be found in different parts of grape (*Figure 2*). The layers of the epidermis and hypodermis are formed by the skin, and the upper part of the pulp cells, and , contain most part of the polyphenols and aromatic compounds.

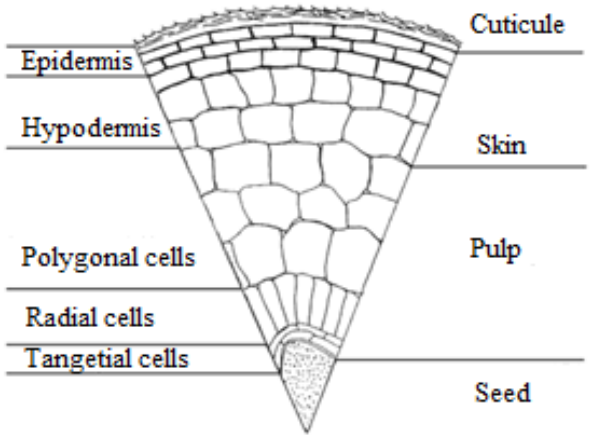

Figure 2 Schematic morphology of grape tissues (adapted from Hidalgo-Togores 2006)

Most of the flavonoids are found in the epidermis, and in the hypodermis cells, including anthocyanin vacuoles and aromatic compounds. Tannins are located in free vacuoles, or in polymeric form connected to the polysaccharides of the cell walls, and also immobilized in the cell wall. The majority of tannin molecules are found in the grape seeds (*Hidalgo-Togores 2006*).

Conventional solid liquid extraction techniques have been widely used in order to gain the antioxidant components from grape marc, or from their macroscopic components, skin and seeds. Various process conditions have been studied and optimized to obtain high extraction

yields with improved extract performance in terms of antioxidant activity and product stability. Different sample preparation processes (drying, milling, degreasing), solvents and solvent mixtures (ethanol, methanol, water, acetone, ethyl acetate, and their combination), and diverse solid:liquid ratios have been studied so far. The effects of pH, extraction temperature and time have also been optimized. Several studies concluded that extraction times between 2 and 5 hours are long enough to obtain high extraction yields at temperatures below 60 ºC, in order to avoid thermal degradation. Acidic pH is beneficial for better stability of phenolics, and the ethanol – water mixture was found to be the best option from an economical point of view, and also considering health aspects (*Lafka et al., 2007, Spigno and De Faveri 2007*).

Quantitative determination of extraction kinetics is necessary for scaling up and process design modelling. Many studies found that polyphenol extraction shows good correlation by first order kinetics. The first order rate equation (*Eq. 1*) can be obtained combining Fick´s second law and the steady-state model taking into account the following assumptions:

» solid particles are considered as flat plates;
» active compound is initially distributed homogenously in the solid;
» the porous solid is considered as a pseudo-homogeneous medium;
» the content of the active compound in the solid varies with time and distance;
» the thermodynamic equilibrium is established at the interface.

$$ln\ (C_e/(C_e-C_t)) = k \cdot t \qquad (1)$$

Equilibrium polyphenol concentration (C_e) is obtained at infinite extraction time ($t=\infty$). The concentration of polyphenols (C_t) at a certain time 't' is readily obtained once the overall rate constant (k) is known. Similar diffusivity coefficients can be found in literature for different grapes and grape residues, which indicates that product nature, origin and environmental conditions are mainly responsible for differences in extraction yields from different samples of the same nature (*Cacace and Mazza 2003, Amendola et al., 2010*).

Although conventional maceration or stirred extraction under mild conditions has been widely studied, it has the ***disadvantage of long processing times*** because of ***slow mass transfer***. Because of this reason, it requires a ***huge and expensive solvent stock***, which must be recovered and removed from the final product. In order to improve the mass transfer and extraction kinetics of antioxidants, different sample ***pre-treatments and novel extraction techniques*** were recently studied and compared, such as supercritical fluid extraction, microwave assisted extraction, Soxhlet-extraction or ultrasound assisted extraction (*Pascual-*

Marti et al., 2001, Martino et al., 2006, Casazza et al., 2010, Rodriguez-Rojo et al., 2012, Peralbo-Molina et al., 2012).

The positive effects of ***microwaves*** and ***ultrasounds*** on polyphenol extraction kinetics from grape marcs have been deeply studied in ***Chapter 4*** and ***Chapter 5*** in comparison to thermal pre-treatment and conventional stirred extraction method, according to a Spanish patent (*Moro-González 2010*). The effect of possible ***thermal degradation*** due to heat treatments is evaluated and discussed in ***Chapter 6***.

2. EXTRACTION PRE-TREATMENTS

2.1. Microwaves application and theory

Electrical ***volumetric heating*** techniques include microwave heating, conduction and induction heating, ohmic heating, and radio frequency heating. In microwave heating, energy is transferred by electromagnetic waves instead of heat flux, as happens in conventional heating processes.

Intensive research on microwave started in the Second World War to develop high-definition radars. In the post-war years the scene of microwave usage extended to heating purposes, either with the appearance of domestic microwave ovens or in industrial applications. Nowadays, it is widely used in the food industry, and in the ceramics, rubber and plastic industry and has been provoking a growing interest for its use in chemical applications (*Meredith, 1998*).

Success behind microwaves is based on the heating theory of the oscillating electromagnetic field, in a ***frequency range between 0.3 and 300 GHz***. Heat is generated by friction of molecules inside the material, due to the reorientation of dipoles in the changing electric field. In industrial and domestic applications a frequency of 2.45 GHz is commonly used, providing 0.94 kJ/mol_{photon} energy with a wavelength of 12.2 cm. The molecule of water, an abundant component in natural raw materials, changes its orientation 10^9 times per second, producing rapid heating. This effect is enhanced by the presence of ionic components and other substances with low specific heat (*Schubert and Regier 2005*).

In addition to the obvious thermal effect of microwaves, a so-called *athermal* or *non-thermal* effect has been reported. This occurs when the alternating electric field of microwaves is able to force the polarized side chains of macromolecules to break their hydrogen bonds, and thus

alter their structure (*Hong et al., 2004; Woo et al., 2000*). The evidence and importance of this phenomenon in natural and organic material processing is under discussion, and was theoretically attacked by Stuerga *(2008*), although contradictory results have been obtained so far. Evidence of an athermal effect is usually claimed or discarded by comparing results from parallel microwave and conventional heating processes. In the case of biogas production, where microwave and conventional heating pre-treatments were compared, contradictory results were found by different authors, showing the results for microwave heating as being either worse (*Climent et al., 2007; Eskicioglu et al., 2006*), better (*Beszédes et al., 2011*), or similar (*Eskicioglu et al., 2007*).

The electromagnetic field and the propagation of microwaves (Figure 3) can be described by ***Maxwell's equations*** (*Eqs. 2-5*), in which electrical phenomena is incorporated. The equations presented here are in vector form for a sinusoidal, time-varying field with angular frequency ($\omega=2\pi f$ *[radian/s]*), and in the case of no electric charge or magnetic dipoles (*Eqs. 2-3*) (*Metaxas and Meredith, 1988*, *Meredith, 1998*)

$$div\ \boldsymbol{D} = 0 \tag{2}$$

$$div\ \boldsymbol{B} = 0 \tag{3}$$

$$curl\ \boldsymbol{E} = -j\omega\boldsymbol{B} \tag{4}$$

$$curl\ \boldsymbol{H} = \boldsymbol{J} + j\omega\boldsymbol{D} \tag{5}$$

Equation (4) corresponds to Faraday's law: the time changing electric field density (*E*) is related to the magnetic field (*B*). The electric field, circulating around a contour, is determined by the rate of change in the magnetic flux through the enclosed surface. Equation (5) describes Ampere's law connecting the magnetic field intensity (*H*) with current density (*J*) and displacement density (*D*). It states that the magnetic field intensity circulating around a contour is determined by the net conduction and displacement current through the enclosed surface.

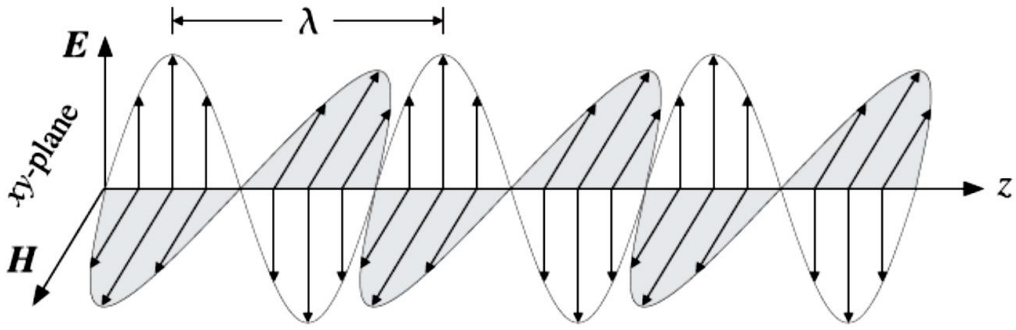

Figure 3. Electromagnetic wave (adapted from Navarrete, 2010)

In addition to the equations above, explaining the interaction between electric and magnetic fields, auxiliary equations are needed to describe the interaction between the material and the electromagnetic fields (*Eqs. 6-8.*):

$$\boldsymbol{J} = \sigma \boldsymbol{E} \quad (6)$$

$$\boldsymbol{D} = \varepsilon_0 \varepsilon' \boldsymbol{E} \quad (7)$$

$$\boldsymbol{B} = \mu_0 \mu' \boldsymbol{H} \quad (8)$$

Where σ is the conductivity of the material. The relative permittivities of the material and of the vacuum are ε' and ε_0 respectively, describing the non-conductive interaction of the material with the electric field. Interaction with the magnetic field is described by the relative permeability of the material, μ', and of the vacuum, μ_0.

In microwave heating, the electric field is considered as the prime source of energy transferred to the workload (*Meredith, 1998*). For this reason, the material interaction with the electric field must be studied, using different workloads, for further design of the optimal microwave process. The ***complex dielectric permittivity*** of the material is determined as follows (*Metaxas and Meredith, 1988*):

$$\varepsilon = \varepsilon_0 \varepsilon_r \quad (9)$$

$$\varepsilon_r = \varepsilon_r' + \varepsilon_r'' \quad (10)$$

In Equation (9), ε_0 and ε_r refer to the free space permittivity and the complex dielectric constant, respectively. Equation (10) shows the real part of the complex dielectric constant (ε_r'), which corresponds to the fraction of ***energy stored in the material*** when an electric field is applied, and the imaginary part (ε_r''), corresponding to the fraction of ***energy which is converted into heat***.

During the microwave process, the material undergoes physical and perhaps chemical changes (moisture content, temperature and salt concentration) which affect its interaction with the dielectric field. These factors must be taken into account when characterizing the dielectric properties of the sample (*Venkatesh and Raghavan 2004*).

Different measurement methods, and the effect of temperature and moisture on the ***dielectric properties of grape marc***, are presented and discussed in ***Chapter 3***.

Industrial microwave systems are mainly composed of three parts: the microwave source, the waveguide and the applicator. In the microwave source ***(magnetron***) the electric energy is converted into microwave energy. The ***waveguide*** conveys the electromagnetic waves to the ***applicator***, to meet the workload with the microwave irradiation. Depending on the material to be treated and on power distribution needs, different applicator types can be used for processing. The most commons are multimode applicators, as in domestic microwave ovens, single-mode applicators and near-field applicator types. In order to achieve high ***power absorption*** in the system, and to avoid back-reflection from the applicator to the source, ***impedance matching*** has to be achieved. For this end, tuning systems are used, which can be controlled during the process and follow the change in the interaction of the material and the dielectric field. Considering these aspects in the microwave design, 95% of the output power from the magnetron can be efficiently dissipated in the workload. Magnetrons may have an efficiency around 85%, relatively to the input electric power. (*Meredith, 1998, Schubert and Regier 2005*). In household microwave ovens and laboratory experimental devices, the impedance matching and good efficiency may not be obtained due to the varying nature of the workload. Nevertheless, thermodynamic considerations can be used as an alternative to quantify the energy absorbed in the experimental setup, since the absolute value of the absorbed energy is so important from an engineering point of view.

2.2. Ultrasounds application and theory

Ultrasounds generate forces of radiative nature including acoustic streaming forces. Ultrasounds are classified into two main categories according to their frequency. High frequencies, in the MHz range, are of low energy and used for diagnostic purposes. The low frequency range, between 20-100 kHz, called power ultrasound, is used to transmit high energy densities. The latter is frequently used in extraction processes, where the generated wave

energy is observed by the solvent, dispersed molecules and particles. However, the density of the propagated energy is lower than 10^{-9} J/atom, which is not high enough to break chemical bonds. A ***cavitation phenomena*** occurs due to ultrasounds. The compression and stretching of the molecular spacing cause the generation and violent collapse of cavitation bubbles in the liquid. The energy thus focused in the sound field is amplified by 11 orders of magnitude, and is therefore enough to break chemical bonds, and even to induce luminescence. ***Stable cavitation*** is obtained at lower power intensities (1-3 W/cm^2), when the bubble size oscillates about an equilibrium value. For intensities higher than 10 W/cm^2, called ***transient cavitation***, the violent collapse of cavitation bubbles occurs after some expansion and compression cycles (*Santos et al., 2009, Zhang et al., 2011*).

The physicochemical effects of ultrasound radiation are due to collapsing bubbles from transient cavitation. They act as micro-reactors of hot gas phase reactions where newly produced active radicals can appear at elevated local temperature and pressure (>1000 °C, 1000 bar), in an intensive mixed gas and liquid mixture. Mechanical effects, like degradation of large molecules, microstreaming of solid surfaces, cell disruption and cell content release depend on the nature of the system (*Santos et al., 2009*).

Ultrasound processes are mainly affected by intensity, frequency, temperature, solvent properties, the presence of dissolved gases and pressure. The ***intensity*** of the ultrasound energy is proportional to the square of the amplitude. There is a required minimum value in order to achieve cavitation in the medium, although excessively high intensities may cause degradation of the desired compounds. Additionally, an excessively high amplitude causes insufficient coupling between the transducer and the liquid, thus decreasing the amount of energy transmitted into the medium, and reducing the operating lifetime of the transducer. In the high ***frequency*** range, the cycles become too short to achieve cavitation in the medium, and so frequency is selected in the kHz range, and usually constant in the process. Increased ***temperature*** has a positive effect on the process, like in the case of extraction, but at the same time causes a less violent collapse of the cavitation bubbles, as they are filled with solvent vapour at higher temperatures. An increase in temperature also affects ***medium properties,*** like viscosity or surface tension. Low surface tension and the use of surfactants enhance the sonochemical effects. Likewise, low solvent viscosity enhances cavitation, but high resistance is showed against the generation of cavitation bubbles in high viscosity liquids. The presence of dissolved ***gases*** or the introduction of gas bubbles also promotes the generation of the

cavitation phenomena, and provide a more uniform energy distribution in the system. Increased external ***pressure*** also promotes sonochemical effects (*Santos et al., 2009, Zhang et al., 2011*).

In US systems, the electrical energy is converted into a high-frequency alternating current (***generator***) and then into mechanical vibrations at a constant frequency (***transducer***), which is then transferred to the medium in an ultrasonic bath or using an ***ultrasonic probe***.

Probe systems deliver ultrasound energy directly into the medium. The design of probes can differ in material (titanium, aluminium, steel) and shape (rod, plate, bar, sphere), depending on the requirements of the process and the material being treated. Besides the advantages of the probe system, there are some drawbacks to take into account. The pitting and erosion of the probe can contaminate the sample, due to direct contact with the product. The strong cavitation field near to the probe tip causes the generation of free radicals, which may negatively affect the material, and also provide non-uniform energy distribution through the vessel.

The use of an ***ultrasonic bath*** does not permit the direct contact with the load. Ultrasound generating elements are placed under the water bath, thus the vibration is conveyed through the water, and then reaches the vessel wall, where the load is found. This technique also suffers from non-uniform energy distribution, moreover the energy absorbed by the material is difficult to determine. Despite its limitation, it is widely used due to the simple design and ease of operation, for example in the surface cleaning of metal parts.

New designs can be found to overcome energy distribution problems. As an example, the use of multifrequency – multimode - modulated ultrasound has been proposed, where two types of oscillation are produced in order to provide uniform distribution and to eliminate standing waves (*Zhang et al., 2011, Santos et al., 2009*).

A wide application spectrum of ultrasounds can be found in emulsification, crystallization, cleaning and surface decontamination, in microbiology fields and in solvent extraction. (*Zhang et al., 2011*)

Oil extraction from herbs was intensified by Ultrasound Assisted Extraction (UAE), with significantly reduced operating time and solvent amount compared to conventional techniques. The microfracture and disruption of cell wall were results of enhanced cavitation, leading to enhanced mass transfer in extraction. In addition, the extraction of proteins, herbal bioactive compounds and polyphenol components was studied. The enhanced extraction mechanism due to increased mass transfer occurs along the high shear forces. The developed macro-turbulence

leads to high velocity inter particle collision and perturbation in micro-porous particles. Therefore, accelerated diffusion takes place besides the mechanical changes in the material, which also positively affect intracellular material liberation (*Vilkhu et al., 2008*).

3. ENERGY BALANCE AND THERMODYAMIC CONSIDERATIONS

"Available energy is energy which we can direct into any desired channel. Dissipated energy is energy, which we cannot lay hold of and direct at pleasure, such as the energy of the confused agitation of molecules, which we call heat. Now, confusion, like the correlative term order, is not a property of material things in themselves, but only in relation to the mind, which perceives them. A memorandum-book does not, provided it is neatly written, appear confused to an illiterate person, or to the owner who understands it thoroughly, but to any other person able to read it appears to be inextricably confused. Similarly, the notion of dissipated energy could not occur to a being who could not turn any of the energies of nature to his own account, or to one who could trace the motion of every molecule and seize it at the right moment. It is only to a being in the intermediate stage, who can lay hold of some forms of energy while others elude his grasp that energy appears to be passing inevitably from the available to the dissipated state." – James Clerk Maxwell

'Diffusion', Encyclopaedia Britannica (1878). In W. D. Niven (ed.), The Scientific Papers of James Clerk Maxwell (1890), Vol. 2, 646

Determination of the efficient energy in chemical engineering processes is of primary importance from the energetic, thus economic point of view. In both, microwave and ultrasound processes, the role of impedance matching, is an indispensable task in equipment and process design in order to obtain optimal energy coupling, thus maximizing the power transfer into the workload and minimizing the reflection from it.

Although the thermodynamic basics for the determination of the absorbed power in the workload are independent from the scale size of the process, its measure is not a standard procedure in the research field. Not only for scaling-up, but also for comparing results obtained from different research groups using different setups, the absorbed energy of the processes should be determined through energy balances. Otherwise, a clear evaluation of the effect of microwave or ultrasound energy on process intensification cannot be performed, as the process is under the influence of experimental setup type, size, geometry and material properties, and non-similar results may be obtained if only the nominal power emitted by the equipment is taken into account. In the following section, an energy balance and the experimental

determination of the absorbed energy will be presented for ultrasound and microwave processes on laboratory scale.

3.1. Heat generation in microwave and ultrasound process

The fraction of electromagnetic ***microwave energy*** absorbed by the sample is subsequently dissipated as heat. This heat is then distributed among the different types of processes that may occur in the sample, according to the thermodynamic state of the material:

» Sensible heat, where the absorbed heat produces an increase in temperature.
» Phase change heat, if transition temperature is reached (boiling, melting …).
» Heat of reaction, if a reaction can take place at the temperature and conditions in the material. In natural products processes, and specifically in the products used in this thesis, this contribution is negligible.

The sensible heat absorbed by the sample is redistributed by thermal conduction and convection, caused by the temperature gradient generated between unevenly radiated zones inside the sample. Heat in the outer part of the material may also be released to the environment if adequate thermal isolation is not provided. On the other hand, also phase change heat must be considered. Particularly, when natural products are processed ***mass losses*** must also be taken into account when open systems are used, as evaporation of volatile components at high temperature is usual for high heating values (*Meredith, 1998*).

When using ***ultrasounds***, the effects produced by the generated heat are analogous to those of microwaves. The majority of the dissipated acoustic energy is converted into heat in the medium, but is not induced within the material, as it is in microwaves. (*Zhang et al., 2011*)

3.2. Energy balance and calorimetric absorbed energy determination

According to the aforementioned heat and mass considerations, absorbed energy ($E_{absorbed}$ *[kJ]*) can be described using an energy balance which takes into account the temperature increment of the system ($Q_{sensible}$), the solvent evaporation (Q_{latent}) and the heat loss into the environment (Q_{loss}) through the vessel wall (*Equation 11*).

$$E_{absorbed} = Q_{sensible} + Q_{latent} + Q_{loss} \qquad (11)$$

Sensible heat ($Q_{sensible}$ *[kJ]*) is calculated from the mass of the material (m_{sample} *[g]*), the temperature increment (ΔT *[K]*) before and after the microwave or ultrasound treatment, and the specific heat capacity of the material (c_p *[kJ/(kgK)]*) (*Equation 12*).

$$Q_{sensible} = c_p m_{sample} \Delta T \quad (12)$$

Latent heat of vaporization (Q_{latent} *[kJ]*) is calculated from the amount of evaporated solvent (m_{vap} *[g])*, measured by weight loss after microwave or ultrasound treatment, and the heat of vaporization of the solvent (ΔH_{vap} *[kJ/kg]*) (*Equation 13*).

$$Q_{latent} = m_{vap} \Delta H_{vap} \quad (13)$$

The ***heat loss*** from the vessel surface (Q_{loss}) was evaluated through the global heat transfer coefficient of the system (*U, [kJ/m^{2}s^{o}C]*) (*Equation 14*)

$$dQ_{loss} = UA(T - T_a)dt \quad (14)$$

, where *A* is the heat transfer area, *T* the temperature of the sample at time *t*, and T_a the ambient temperature. The value of the constant *UA* product was calculated in a separate experiment where the decrease of temperature over time of a hot sample was recorded after finishing the microwave or ultrasound treatment. Heat loss from the surface of the vessel containing the sample was related through a differential heat balance (*Equation 15*):

$$m_{sample} c_p dT = -UA(T - T_a)dt \quad (15)$$

Where m_{sample} *[g]* is the sample mass, c_p *[kJ/(kgK)]* is the specific heat of the sample, *T [^{o}C]* is the temperature in the vessel at *t* cooling time, and T_a *[^{o}C]* is the ambient temperature. By integrating Equation (15) between the initial (T_0) and final (T_f) temperatures of the sample after a certain cooling time (t_f), the following expression is obtained:

$$UA = \frac{m_{water} C_p}{t_f} ln \frac{T_0 - T_a}{T_f - T_a} \quad (16)$$

Equation (14) was integrated assuming linear temperature increase up to the boiling temperature of the sample (T_b), and constant boiling temperature during the subsequent evaporation (*Equation 17*):

$$Q_{heat\ loss} = UA \left[\left(\frac{T_0 + T_b}{2} - T_a \right) t_b + (T_b - T_a)(t_f - t_b) \right] \quad (17)$$

Where t_b is the time required to reach boiling temperature, and t_f is the total microwave treatment time.

A schematic temperature profile is presented (Figure 4) to clarify the experimental implementation of the calorimetric determination method:

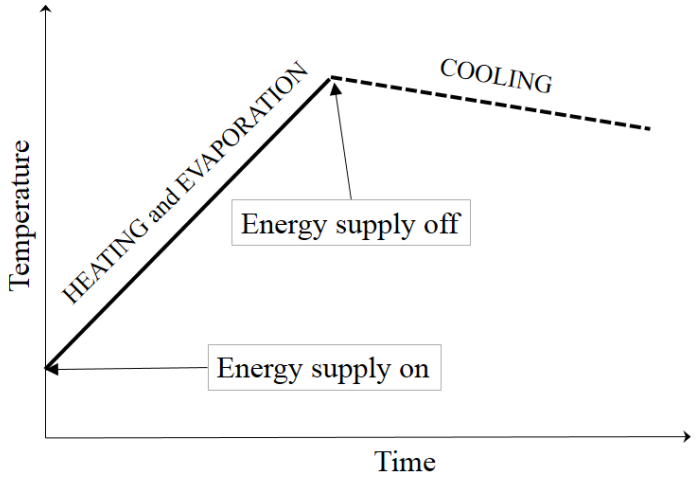

Figure 4. Schematic temperature profile in calorimetric energy determination

3.3. Emitted energy and equipment efficiency

The energy amount provided by the microwave generator ($E_{emitted}$) can be calculated as the definite integral of the applied power (P *[W]*) over t treatment time (*Equation 18*) (*Meredith, 1998*).

$$E_{emitted} = \int_0^t P dt \qquad (18)$$

For nearly constant power processing, the emitted energy is calculated by the product of the nominal power of the oven, and the treatment time.

In the case of ultrasound, the intensity (I *[W/cm^2]*) is proportional to the square of the amplitude, but cannot be directly determined from the experimental settings, as the device only allows the amplitude applied to be fixed as a percentage of the maximum at a constant frequency. Therefore, the power consumption must be measured during the process to determine the emitted energy by integration over time.

From the emitted energies thus calculated in the process, the equipment efficiency can be obtained as the relationship between absorbed and emitted energy (*Equation 19*).

$$\eta\ (\%) = \frac{E_{absorbed}}{E_{emitted}} \cdot 100 \qquad (19)$$

Equipment efficiency and absorbed energy calculations are considered in all chapters of the thesis, where ***microwave or ultrasound*** treatments were involved. Depending on the experimental device and final treatment temperatures, the latent heat and heat loss can be neglected compared to the sensible heat amount.

4. CONCLUDING REMARKS

Microwave and ultrasound treatments appear to be promising techniques in order to enhance process kinetics, where cell disruption is the rate-limiting step, as in the cases of wastewater sludge disintegration, and extraction processes from microalgae or grape marc. However, both techniques involve the use of electric energy, and additional equipment and operating costs. A negative economic balance may override all the advantages of these extraction techniques. The longer the extraction time required, the greater the size of the equipment and the associated cost. For this reason, the proposal of effective and short pre-treatments on the natural material, as an additional preliminary stage in the conventional process line, may be a better alternative to the development of a completely new approach. The hereby intensified process kinetics could achieve reduction, not only in power or in total energy demand, but also in processing time, equipment size and solvent stock, thus greatly reducing the overall cost of the process.

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Chapter 1.

The influence of energy absorbed from microwave pre-treatment on biogas production from secondary wastewater sludge

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Bioresource Technology 102 (2011) 10849-10854.

THE INFLUENCE OF THE ENERGY ABSORBED FROM MICROWAVE PRE-TREATMENT ON BIOGAS PRODUCTION FROM SECONDARY WASTEWATER SLUDGE

Abstract

In this study, microwave treatment is proposed as a way to accelerate the hydrolysis in anaerobic digestion of municipal wastewater sludge. The influence of the absorbed energy, power and athermal microwave effect on organic matter solubilization and biogas production has been studied. In addition, a novel method that considers the absorbed energy in the microwave system is proposed, in order to obtain comparable experimental results. The absorbed energy is calculated from an energy balance, considering the sum of sensible and latent heats, and heat loss from a sample surface.

The highest solubilization was achieved using 0.54 kJ/ml at 1000W, where an increment of 7.1% was observed in methane production, compared to the untreated sample. Using a higher energy value (0.83 kJ/ml), methane production further increased (to 15.4%), but solubilization decreased. No power influence was found when 0.54 kJ/ml was applied at 1000, 600 and 440 W. Microwave heating was compared to conventional heating in two different experimental setups, providing similar methane yields in all cases.

1. INTRODUCTION

Municipal waste water treatment plants generate large amounts of excess sludge which must be disposed of. This is a problem of growing importance, representing up to 50% of the current operating costs of a wastewater treatment plant (*Appels et al., 2008*).

Most plants use anaerobic digestion to stabilize the organic material of this sludge and reduce its biomass. In addition, it produces biogas and improves the dewatering properties of the residual sludge (*O'Flaherty et al., 2006*). The anaerobic degradation of the sludge is a three-step sequence: 1) hydrolysis, 2) acidogenesis, and 3) methanogenesis, in which the former, hydrolysis, is accepted as the rate-limiting step. Sludge particles are essentially concentrated aerobic microbial cells. In order to be hydrolyzed, anaerobic bacteria must release extracellular enzymes that break down and solubilise the rigid microbial cell walls (*O'Flaherty et al., 2006; Appels et al., 2008*). In order to increase the biodegradability of the sludge, various mechanical, ultrasonic, chemical and thermal pretreatment methods have been proposed (*Perez-Elvira et al., 2006*). The pretreatment by irradiation of the sludge using microwaves has been one of the methods reported repeatedly in literature over the last decade.

Microwaves can improve the rupturing of the cell wall, thus enhancing anaerobic hydrolysis in two different ways. Firstly, the *thermal effect* corresponds to degradation caused by temperature increase. Also, the internal heating and evaporation of the intracellular water causes an increase in internal pressure that can lead the cell wall to rupture (*Lyons and Hatcher, 1972; Golmakani et al., 2008; Lucchesi et al., 2007; Uquichea et al., 2008*). Secondly, the so-called *athermal* or *non-thermal effect* must be considered. This occurs when the alternating electric field of microwaves is able to force the polarized side chains of the cell wall macromolecules to break their hydrogen bonds, and thus alter their structure (*Hong et al., 2004; Woo et al., 2000*).

Several studies have been reported investigating optimal conditions (power, treatment time and temperature) for microwave wastewater sludge treatment with the aim of solubilizing organic matter or enhancing anaerobic digestion. In some of these papers, microwave treatment is compared with other thermal or ultrasonic processes in order to assess its potential as a sludge disintegration method (*Beszédes et al., 2009, Beszédes et*

al., 2011, Climent et al., 2007, Eskicioglu et al., 2006, Eskicioglu et al., 2007, Guo et al., 2008). However, the results reported in these studies lead to conflicting results:

- ***Effect of power on organic matter solubilization*.** Organic matter solubility is calculated from the COD (chemical oxygen demand) of the soluble phase. According to some authors, the use of higher power during microwave treatment leads to lower solubilization (*Eskicioglu et al., 2007; Park et al., 2010; Toreci et al., 2009*), while others report the opposite effect (*Climent et al., 2007*).
- ***Effect of power on biogas production***. Similar (*Eskicioglu et al., 2007*) and higher (*Toreci et al., 2009*) biogas production were found with increasing microwave power.
- ***Athermal effect***. The presence of an athermal effect is usually determined through the comparison of microwave heating and conventional heating processes. The biogas production reported in these studies, where microwave instead of conventional heating was used, was in some cases lower (*Climent et al., 2007; Eskicioglu et al., 2006*), in another case higher (*Beszédes et al., 2011*), and in a last one it remained unchanged (*Eskicioglu et al., 2007*).

The use of different raw materials can lead to different quantitative results. However, the reason for these contradictory conclusions can be explained due to the difficulty in comparing results using different experimental conditions in microwave treatments. Only a fraction of the power applied in microwave heating is absorbed by the sample material. This fraction depends on factors such as dielectric properties, the size and geometry of the sample, microwave frequency and intensity, process time, and oven cavity characteristics (*Swain and James, 2005; Campañone and Zaritzky, 2005; Gunasekaran and Yang, 2007; Zhu et al., 2007*). In order to analyze and compare results from several experiments it is necessary to determine the fraction of energy absorbed by the sample.

The triple objective of this study was, firstly, to develop an experimental procedure in which absorbed microwave energy could be calculated from an energy balance; secondly, to evaluate the influence of absorbed microwave energy and power on treated sludge; and thirdly, to reveal the presence of a possible athermal effect. The influence of absorbed

energy and power was evaluated by measuring the change in organic matter solubilization and biogas production. The athermal effect was investigated by treating equivalent sludge samples through both microwave and conventional heating to boiling point for the same period of time, and comparing the results in terms of biogas production.

2. MATERIALS AND METHODS

2.1. Secondary wastewater sludge

Samples of secondary wastewater sludge were provided by the Municipal Wastewater Treatment Plant of Valladolid (Spain). The decanted sludge was further centrifuged at the treatment plant up to an approximate 7-8 w/w% of total solids, to eliminate the external water. Under these conditions, it was predicted that the cell wall rupture would be more easily achieved, since most microwave radiation would be absorbed by intracellular water, causing the gradient of pressure by rapid evaporation. The effect of microwave radiation being absorbed by extracellular water is only supposed to make a contribution of a thermal effect. Concentrated sludge was stored at 5ºC and used for experimental work within 2 days.

2.2. Experimental methods and equipments

2.2.1. Microwave treatment

Microwave treatments were performed in a domestic microwave oven (Figure 1) (Panasonic NNGD-566M; oven cavity: 359 x 352 x 217 mm) where time and power (250; 440; 600; 1000W) could be selected (1). The oven had previously been adapted for laboratory purposes: the grill apparatus was taken out and a vapor outlet exit tube was welded (2) onto the top of the oven. Radiation leakage was measured from a distance of 1 cm up to 1 m from the working oven to ensure that electrical field did not reach 5 W/m^2. A turning cylindrical carousel (3) was designed to hold the sludge samples in 1-6 tubes (Nalgene) (4) and to allow the vapor produced to leave (5) the microwave oven to condense (6) and be collected (7). Three tubes were used in the experiments with 30 g of sludge in each one. In order to promote cell wall rupture, experiments were performed at

ambient pressure. Working at higher pressures would allow for operating at higher temperatures, but could also render more difficult the cell wall rupture by intracellular water evaporation.

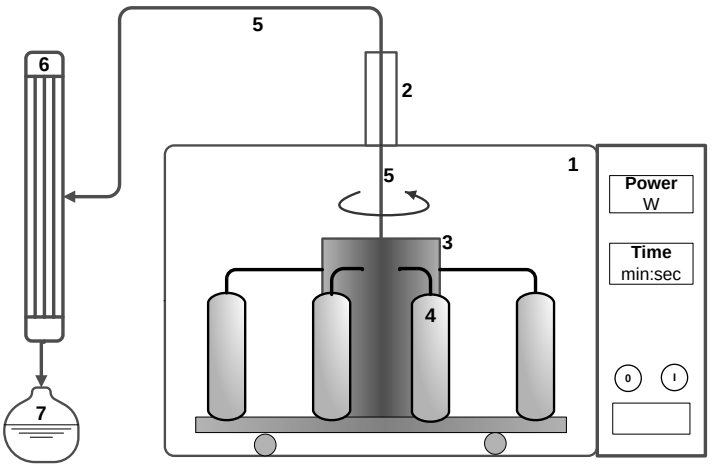

Figure 1 *Microwave oven for sludge treatment: 1 – microwave oven; 2 - welded tube on microwave cavity; 3 – turning carousel with vapor collection (r = 160 mm); 4 – Polycarbonate tubes (85 ml, d= 38 mm); 5 – silicon tube for vapor exit; 6 – condenser; 7 – condensed water collection.*

After exposing the sludge to microwave radiation, evaporated water was weighed and refilled into the tubes to maintain the TS (total solid) content in the samples. Temperature was recorded before and after treatment.

The effect of microwave energy on sludge was measured in two different sets of experiments: 1) experiments at constant power (1000 W) with different treatment times, and 2) experiments at constant absorbed energy (0.54 kJ/ml) at 1000, 600 and 400 W microwave powers (Table 1). To increase the repetitivity of experiments, the magnetron was warmed-up by heating 500 ml water for 5 minutes in the oven before the experiments.

An additional experimental setup was also applied in the same microwave oven for further comparison with conventional heating. In order to keep a constant water content in the sample during microwave treatment, the turning carousel was replaced by a cylindrical Pyrex vessel (d = 140 mm), and the vapor produced was condensed and refluxed during the treatment. Sludge sample height was chosen around 1.5 cm, considering the penetration depth of the microwaves. In this refluxed experimental setup, 150 g of preheated sludge was treated at 250 W for 12 minutes.

Table 1 *Microwave treatment conditions and Energy distribution in microwave treatments (kJ/ml)*

	Power (W)	Time (min)	$E_{emitted}$ (kJ/ml)	$Q_{sensible}$ (kJ/ml)	Q_{latent} (kJ/ml)	$Q_{heat\ loss}$ (kJ/ml)	$E_{absorbed}$ (kJ/ml)	Efficiency (%)
1	1000	0.8	0.53	0.35	0.01	0.05	0.41	77
2	1000	1.2	0.80	0.39	0.07	0.08	0.54	66
3	1000	2.0	1.33	0.39	0.36	0.08	0.83	62
4	600	2.0	0.80	0.39	0.13	0.08	0.60	75
5	440	2.7	0.80	0.39	0.11	0.10	0.60	75

2.2.2. Convective heat treatment

For convective heating purposes, two types of heating sources were used in experiments, and later compared to corresponding microwave treatments. In the first one, an electric mantle (J.P. Selecta; 410 W at 80%) was used, and 400 g of sludge was heated up from 7ºC to 85ºC in 10 minutes, while being stirred continuously.

In the second case, a 100ºC water bath was used to heat up 300 g of sludge for 50 minutes, from 8ºC up to boiling point (97ºC). Next, the sample was divided into two portions: 1) 150 g of sludge was taken out for microwave treatment, and 2) the rest of the sample was further heated in the water bath for an additional 12 minutes. During the heating process the sample was often stirred. In both the conventional and microwave heating processes, the temperature of the sample remained constant, as it was maintained at boiling point.

In Table 2, experimental conditions are summarized for the comparison between conventional heating and microwave treatments.

Table 2 *Experimental conditions for the comparison of conventional heating (CH) and microwave treatment (MW)*

Sample	Preheating	Power (W)	time (min)	T $_{initial}$ (ºC)
MW1	-	1000	1.2	7
CH1	-	330	10	7
MW2		250W	12	boiling
CH2	Water bath 100ºC; 50 min	Water bath	12	boiling

2.3. Measurement of microwave oven efficiency

Absorbed energy is reported here in kJ per volume of sludge samples. It is also usual to provide this value in terms of grams of total solids *(Beszédes et al., 2009; Ahn et al., 2009*). However, most of the microwave energy is absorbed by water present in the system.

Absorbed energy ($E_{absorbed}$ *[kJ/ml]*) by sludge sample was calculated by an energy balance of three contributing factors:

$$E_{absorbed} \approx Q_{sensible} + Q_{latent} + Q_{heat\ loss} \quad (1)$$

Sensible heat ($Q_{sensible}$ *[kJ/ml]*) is calculated from the mass of sludge sample (m_{sludge} *[g]*), the sludge temperature increment (ΔT *[ºC]*) and the specific heat capacity of water ($C_{p,w}$= *4.1855 [J/(g·K)]*):

$$Q_{sensible} = m_{sludge}\, C_{p,w}\, \Delta T \quad (2)$$

Latent heat of vaporization (Q_{latent} *[kJ/ml])* is calculated from the amount of evaporated water (m_{vap}), measured by weight loss after microwave treatment, and water heat of vaporization (ΔH_{vap}= *- 2,270 [kJ/kg]):*

$$Q_{latent} = m_{vap}\, \Delta H_{vap} \quad (3)$$

Finally, heat loss ($Q_{heat\ loss}$) from a sample vessel was determined using the global heat transfer coefficient (U):

$$dQ_{heat\ loss} = - UA(T-Ta)dt \quad (4)$$

Where *A* is the heat transfer area, *T* is the temperature of the sludge sample and *Ta* is the ambient temperature. The value of the constant *UA* product was calculated in a previous experiment from the temperature decrease of three water filled tubes which were cooled in the microwave oven to room temperature in the turning carousel, without microwave irradiation. Heat loss from the tubes and water cooling were related through a differential heat balance:

$$m_{water}\, C_{p,w}\, dT = - UA(T-Ta)\, dt \quad (5)$$

where m_{water} is the amount of water inside tubes (3x30 g), $C_{p,w}$ is the specific heat of water, *T[ºC]* is water temperature in the tubes, *t* is cooling time and *Ta [ºC]* is the ambient temperature. By integrating this equation between the initial (T_0) and final (T_f) temperatures after a certain cooling time (t_f):

$$UA = \frac{m_{water} C_{p,w}}{t_f} ln \frac{T_0 - T_a}{T_f - T_a} \quad (6)$$

Equation 6 was integrated assuming linear temperature increase up to boiling temperature of sludge (T_b), and constant boiling temperature during subsequent evaporation:

$$Q_{heat\ loss} = UA\left[\left(\frac{T_0+T_b}{2} - T_a\right) t_b + (T_b - T_a)(t_f - t_b)\right] \quad (7)$$

where t_b is the time required to reach boiling temperature, and t_f the total microwave treatment time.

Energy emitted ($E_{emitted}$ *[kJ/ ml]*) was calculated from selected power and time in the microwave oven. Efficiency (η_{mw}) was calculated for all experiments as the ratio of absorbed ($E_{absorbed}$) to emitted ($E_{emitted}$) energy:

$$\eta_{\mathrm{MW}} = \frac{E_{absorbed}}{E_{emitted}} \quad (8)$$

2.4. Sample characterization and analytical methods

All sludge samples were analyzed before and after the microwave pretreatments. Total and volatile solids (TS, VS) and total and soluble chemical oxygen demand (COD_T, COD_S) were measured (Table 3) by Standard Methods (Eaton *et al*., 2005). The soluble phase was obtained by centrifugation at 15,500 g for 5 minutes; the supernatant was filtered through filters of 0.45 μm (Millipore) and stored at 4ºC until further analysis.

Solubilization of organic matter was calculated according to (*Tang et al., 2010*)

$$S = \frac{(\frac{COD_S}{COD_T})_{treated} - (\frac{COD_S}{COD_T})_{initial}}{(\frac{COD_S}{COD_T})_{initial}} S = \frac{(COD_s / COD_t)_{treated} - (COD_s / COD_t)_{initial}}{(COD_s / COD_t)_{initial}} \quad (9)$$

Where *S* is the organic matter solubilization, and COD_S/COD_T is the ratio between soluble and total chemical oxygen demand for treated *(treated)* and untreated *(initial)* sludge.

Table 3 *Raw material characterization: solid content and chemical oxygen demand*

Sludge Characteristics	
TS (%)	7.17 ± 8%
VS (%)	5.39 ± 6%
CODt (mg O_2/l)	74067 ± 5%
CODs (mg O_2/l)	2307 ± 19%
sludge age (days)	15

Metal (Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) content was determined from the total and soluble phases by inductively coupled plasma atomic emission spectroscopy. (Varian, 725-ES)

The protein content of the soluble phase was measured by the Lowry method to investigate the cell rupture effect of treatments (*Bio-Rad DC Protein Assay Kit with bovine serum albumin standard*).

Biogas production was determined under mesophilic conditions (35ºC). Samples were inoculated with anaerobic sludge from wastewater bioreactors in 125 ml-bottles, in triplicate. Substrate/ inoculum ratio was chosen as 0.5 of the volatile solids *(g/g)*. The evolution of headspace pressure was measured in order to quantify the biogas, and its composition was analyzed by gas chromatography (Varian CP-3800, USA) with a thermal conductivity detector (TCD), applying helium as the carrier gas. Accumulated methane volume (35ºC) was calculated per gram of added volatile solids (mL CH_4/g VS).

3. RESULTS AND DISCUSSION

3.1. Energy efficiency measurements

Equipment efficiency varied between 62 and 77% depending on the process variables (Table 1). Heat loss contribution was in the range of 10-16% of absorbed energy, increasing with the treatment time. Sensible heat was the main contribution (≈86.5%) to absorbed energy, when boiling temperature was not achieved. When water evaporation occurred, this percentage was divided between the sensible and the latent heat, depending on the amount of evaporated water.

3.2. Microwave energy effect on solubilization and biogas production

Using a constant power (1000 W), different treatment times were used in order to apply a wide range of absorbed energy values to the sludge samples. During the treatment, the experimental setup remained opened to atmosphere, therefore the sample temperature only increased up to the boiling point, when water evaporation started at a constant temperature.

Figure 2 shows the results of COD solubilization and protein concentration versus the absorbed microwave energy. At low absorbed energies, the solubilization values, as well as the protein concentrations, increased. When the absorbed energy was around 0.4-0.5 kJ/ml the sludge reached its boiling point, and the maximum values of solubilization and proteins were obtained.

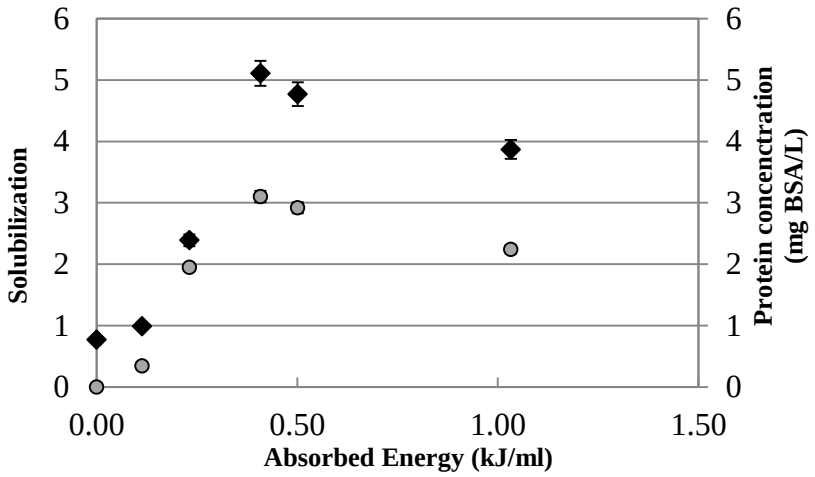

Figure 2 *(●) : COD solubilization (Eq. 6), and (♦) : protein concentration of microwave treatments at constant power (1000 W) in a wide range of absorbed energy.*

Beyond this value, water evaporation took place at a constant temperature, and solubilization and protein concentration showed a slightly decreasing pattern. Although the evaporated water was replaced after treatments, for high values of absorbed energy, the evaporation partially dried the sludge samples during the treatment, and therefore sludge floc agglomeration may have occurred. Part of the liberated soluble matter could have been blocked inside the agglomerates, and later rehydration using the evaporated water was not enough to solubilise them. Antoher reason for the COD solubilization decreasing could be the loss of volatiles during the water evaporation. As previously explained, experiments were performed at ambient pressure to allow for cell wall rupture by intracellular water evaporation.

From these experiments, an approximate value of 0.4-0.5 kJ/ml can be established as the optimal absorbed energy for organic matter solubilization, when the sludge sample reached its boiling point.

Metal content measurements (Table 4.) showed that slight liberation occurred in the case of Al, Ni, and Zn content in the soluble fraction. Fe content slightly decreased with higher energy treatment.

Table 4 *Metal content (mg/kg) of total and soluble fractions before (Control) and after microwave treatment at 1000W for 0.7 and 1.3 min.*

	Al	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Control $_{soluble}$	0.564	<0.40	<0.40	<0.40	15.5	<0.40	<0.40	<0.40	0.478
0.7 min $_{soluble}$	2.79	<0.40	<0.40	<0.40	14.7	<0.40	0.529	<0.40	2.11
1.3 min $_{soluble}$	2.72	<0.40	<0.40	<0.40	13.5	<0.40	0.496	<0.40	1.91
Control $_{total}$	701	0.083	2.14	10.1	795	5.41	2.11	3.08	66
0.7 min $_{total}$	641	0.087	2.07	10.3	774	5.47	2.15	2.96	66
1.3 min $_{total}$	716	0.091	2.25	10.8	836	5.71	2.25	3.24	70

Among these metals, zinc and nickel are the principal elements limiting sludge recycling to agricultural land. However, a low level of nickel would not affect the bioprocess (*Alvarez et al., 2002*). Guo *et al.*, (*2008*) reported that microwave sludge treatment can liberate heavy metals, and that elevated Cu content inhibits the H_2 production in anaerobic digestion. However, this effect was more serious in the case of ultrasonication and could not be observed in the sterilization treatment. In their experiments, 67.2 kJ microwave energy was used for an unreported quantity of sludge, thus it cannot be compared to the treatments performed in this study. In order to ensure against possible inhibition risks during the digestion process, a screening of separate metal forms by sequential extraction has been proposed in literature (*Alvarez et al., 2002*).

After organic matter solubilization measurements, biogas production was determined for experiments 1, 2 and 3 (Table 1), and compared to one untreated sample. As shown in Figure 3, the final CH_4 production was 195 ±7 ml/g added volatile solids in the case of the untreated control sample. For treated samples, production yields were 206 ±4 ml, 209 ±11 ml and 225 ±6 ml for *experiments 1*, *2* and *3* respectively. Although the highest biogas production was obtained in the case of the highest absorbed energy treatment, it can be observed that the time required to produce as much biogas as the control in 22 days was reduced by 20% in *experiments 1* and *2*, and by 50% in experiment 3. The decrease of the

residence time in digestion would allow for the use of smaller digestion tanks, so reducing the capital and operational costs of the process.

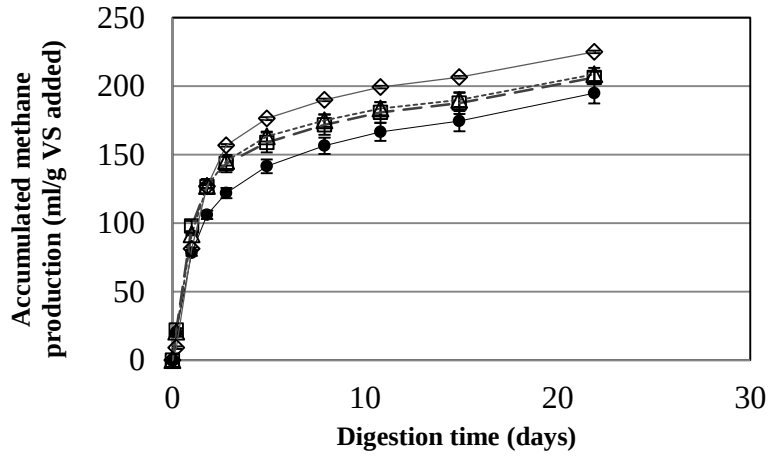

Figure 3 *Accumulated CH4 production (ml/g VS added) for control and treatments with 0.40, 0.54 and 0.83 kJ/ml absorbed energy at constant power (1000 W). (-●-): Control, untreated sample; (- -□- -): 0.40 kJ/ml; (••Δ••): 0.54 kJ/ml; (--◊--): 0.83 kJ/ml*

Although *experiment 2* (0.54 kJ/ml) was the optimum for the organic matter solubilization, biogas production tests did not confirm this result, since biogas yield continued to increase with absorbed energy in microwave treatment, even beyond the boiling point. Although the soluble fraction was not positively affected over the boiling point, further structural changes in sludge flocs could have occured, having a positive effect on the anaerobic digestion. In the case of the untreated sample, the methane content in biogas composition was 45.10±0.46 % on the first day, increased up to 67.30±0.91 % on the second day and stabilized at 62.94±1.01 % during the 20 days of production. Microwave treatments did not changed the methane yield in biogas composition, indicating an undisturbed bioprocess in biogas production.

In the study of Beszédes *et al*., (*2009*) microwave treatment was used in dairy sewage sludge to enhance anaerobic digestion. The 5 W/g treatments resulted in the same solubilization as 10 W/g, however 10 W/g treatment had a significantly higher biogas yield. Higher energy in closed vessels resulted in higher treatment temperature, thus, enhanced biogas generation was observed (*Eskicioglu et al., 2009*). Tang *et al.*, (*2010*) studied the treatment energy required to obtain maximum biogas production with microwaves. In their study, the experimental setup worked up to the boiling temperature

of sludge (≈100ºC), as in this manuscript. The maximum organic matter solubilization was reached at the boiling point with a specific energy of 0.12 kJ/ml, four-fold lower than the energies used here (0.4-0.5 kJ/ml). The energy required to boil the sludge sample depends on their initial temperature, which could explain such a difference between the results mentioned.

3.3. The effect of microwave power on solubilization and biogas production

In this set of experiments, a value close to 0.54 kJ/ml of absorbed energy was used at 1000, 600 and 440 W with 1.2; 2 and 2.7 min treatment times, respectively. The obtained COD solubilization and protein concentration results can be seen in Figure 4. Allowing for an error of 3 and 4% in solubilization data and protein concentration, respectively, no clear differences can be observed between the experiments with microwave treatment.

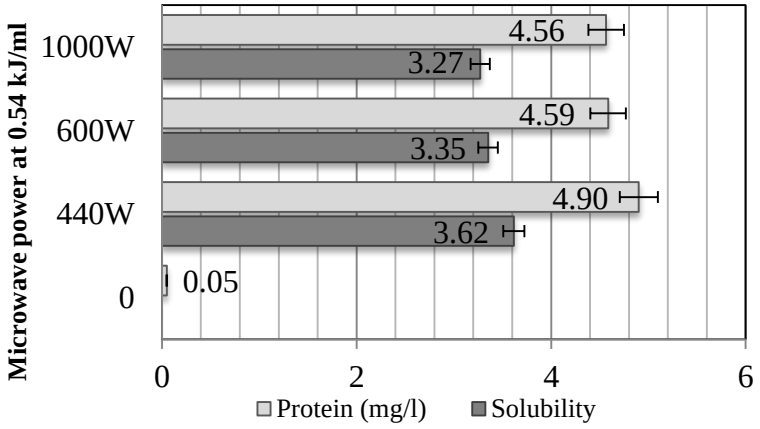

Figure 4 *COD solubilization results and protein concentrations results using 0.54 kJ/ml absorbed energy treatment with 1000, 600, 440 W and control sample. (■): Solubilization; (■): Protein concentration (mg BSA/L)*

Similar COD solubilization was also found by Chang *et al*., (*2011*) when waste activated sludge was treated by microwaves at 300, 450 and 600 W until sludge boiling occurred at 80ºC. Park *et al*., (***2010***) found considerably higher solubilization values, using 400 W instead of 1600 W at 60, 90 and 120ºC. The same solubilization tendency was observed using lower microwave intensity at a higher temperature (175ºC) (*Toreci et al., 2009*), and also at lower temperatures (50, 75 and 96ºC) (*Eskicioglu et al., 2007*). These results

suggest that differences in treatment time may be responsible for better solubility results, since longer processing times are required at lower powers to reach the same final temperature. On the other hand, Climent *et al.*, (*2007*) obtained higher solubilities at 800 W than at 400 W, with 13000 kJ/kg suspended solids (equivalent to 0.48 kJ/ml sludge). However, in the same study, higher solubility values were obtained at 400 W with a sample of 7800 kJ/kg suspended solids. Despite these contradictory results, the present study did not find clear differences in COD solubilization at different power levels. It is probable that the difference between the results may be caused by the alteration of the energy absorption in the experimental setup.

After the analysis of the organic matter solubilization, the biogas production was measured. In Figure 5, the accumulative biogas production can be seen for raw sludge and for microwave treated samples. All treatments with 0.54 kJ/ml enhanced the methane yield (211±11 ml/g VS $_{added}$), compared to the untreated sludge (195±7 ml/g VS $_{added}$), but almost identical methane production curves were obtained at all power levels. This confirms the observation drawn from COD solubilization, that absorbed energy as a specific treatment condition plays a greater role than the applied power.

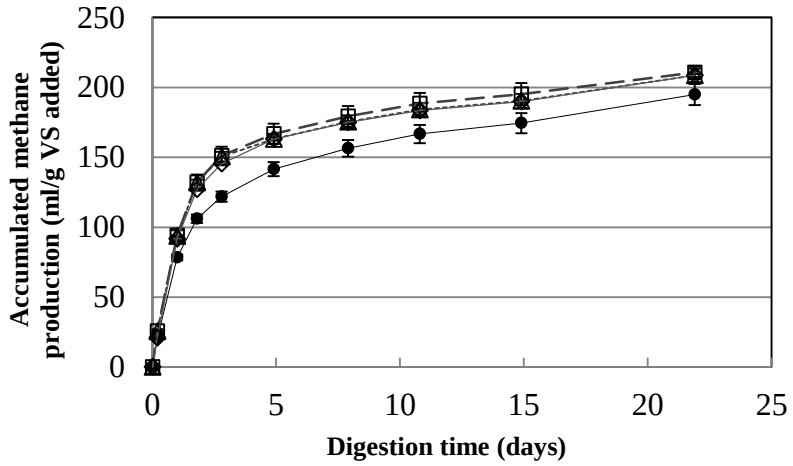

Figure 5 *Accumulated CH4 production (ml/g VS added) for power effect experiments at 0.54 kJ/ml. (-●-): Control, untreated sample; (- -□- -): 440 W; (••Δ••): 600 W; (--◊--): 1000 W*

Toreci *et al.*, (*2009*) reported higher biogas production at higher microwave intensity; however, the solubilization was better at lower power levels. There is a lack of information about the effect on biodegradability at different microwave powers in other

studies, usually focusing on organic matter solubilization for this analysis (*Climent et al., 2007; Eskicioglu et al., 2007; Park et al., 2010*).

3.4. Microwave athermal effect

In a first set of experiments (Table 2) COD solubilization results were 3.27 and 5.26 for microwave (MW1) and conventional heating (CH1) respectively. The same behavior was observed by Climent *et al*., (*2007*) for high and low temperature treatments (135ºC and 70ºC) with conventional heating versus microwave treatment. Eskicioglu *et al*., (*2007*) obtained the trend observed here, working at lower temperatures (50, 75, 96ºC). However, Beszédes *et al*., (*2009*) observed lower biological oxygen demand solubilization at 95ºC with convective heat treatments than with microwaves. It is believed that through conventional heating over a longer time period, more solubilization can be achieved, while the rapid microwave treatment does not allow time enough for organic matter liberation in the process (*Eskicioglu et al., 2006*). For this reason, here, a second experimental setup is proposed to compare conventional heating to the microwave treatment.

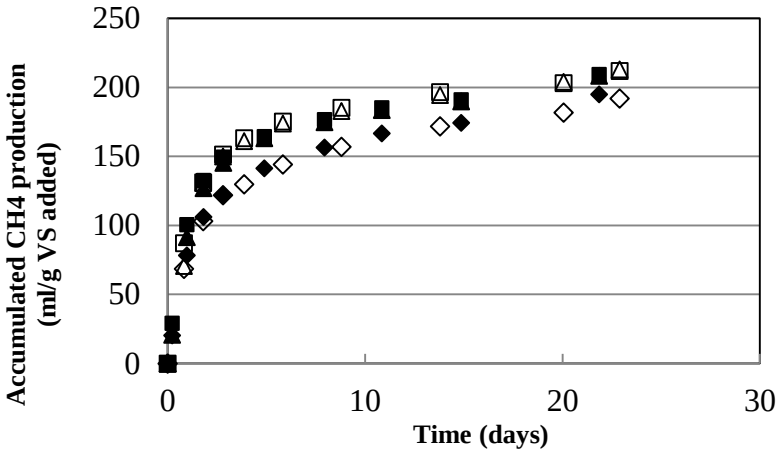

Figure 6 *Accumulated CH4 production (ml/g VS added) for compared experiments with conventional and microwave heating. (♦): Control 1, untreated sample; (▲): MW 1; (■): CH 1; (◊):Control 2, untreated sample; (Δ): MW 2; (□): CH 2.*

In that set of experiments (MW2 and CH2), equal processing times and treatment temperatures were applied in order to reveal a possible athermal effect when using microwaves. COD solubilization was 22.45 and 20.07 for MW2 and CH2 respectively.

Apart from the identical temperature and treatment times in both experiments, in the case of MW2, continuous vapor reflux was performed to avoid the agglomeration of sludge flocs due to water evaporation during treatment. These differences indicate that the comparative experiments need to be similar in as many operating conditions as possible to obtain comparable results.

In the biogas production test, the same tendency was observed for both the experimental series (Figure 6). MW1 and CH1 resulted in 208±12 ml/g VS_{added} and 203±1 ml/g VS_{added} methane production yield respectively, while 195±7 ml/g VS_{added} methane yield was observed with untreated sludge (Control 1). A further increase in production can be seen in the second set of experiments when compared to its control (Control 2: 192±2; CH2: 212±4; MW2: 213±1), but now there is no clear difference between microwave treated and conventionally heated sludge. Higher biogas generation was reported (*Eskicioglu et al., 2007*) for microwave treated samples at 50, 75 and 96ºC compared to the conventionally heated sludge, although in an earlier study (*Eskicioglu et al., 2006*) the conventional heating at 96 ºC was more effective than microwaves in terms of biogas yield.

From the results of the present study, no evidence of the athermal effect of microwaves was found, since similar biogas production was obtained using the conventional heating sludge pretreatment.

4. CONCLUSIONS

» In microwave treatments experiments, the absorbed energy must be reported as a fundamental operating variable, and used when comparing operating conditions.

» The highest value in COD solubilization was obtained using 0.54 kJ/ml of absorbed energy; however, the highest increment in methane production (15%) was achieved with the highest value used (0.83 kJ/ml).

» Using the same absorbed energy, different power levels did not give conclusive COD solubilization results; however, there was no difference in methane production when the power lever was changed.

» No evidence of the athermal effect of microwaves was found, as similar biogas production results were obtained with microwave treatment and conventional heating.

ACKNOWLEDGEMENTS

The authors wish to thank the Junta de Castilla y León for their financial support (Project GR11-2008).

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Chapter 2.

Intensification of lipid and pigment extraction kinetics by microwave pre-treatment on microalgae

INTENSIFICATION OF LIPID AND PIGMENT EXTRACTION KINETICS BY MICROWAVE PRE-TREATMENT ON MICROALGAE

Abstract

Both bioactive compounds and the production of biodiesel from microalgae are of great industrial interest nowadays. Among the multiple difficulties in processing microalgae, cell disintegration is always a challenge to overcome. For this purpose microwave energy can be applied, allowing accelerated heating of the algae´s intracellular water. In this study, solvent free microwave pre-treatment (SFMP) was suggested as a way to enhance the extraction kinetics of lipids and pigments from *Nannochloropsis gaditana* and *Scenedesmus almeriensis,* respectively. The pre-treatments enhanced the Soxhlet-extraction of dry biomass and the liquid-liquid extraction kinetics, although the latter did not allow a proper study of the different variables of the pre-treatment, due to the slow mass transfer between the organic and aqueous phases. Cell surface damage was also observed using scanning electron microscope. Taking into account the absorbed microwave energy, SFMP was found to be successful in diverse experimental conditions for downstream process intensification.

1. INTRODUCTION

The interest in microalgae strains from a biotechnological point of view has grown over the last decades. As photosynthetic organisms, they convert CO_2, water and light into complex organic compounds of industrial interest. These include biofuels such as biogas (the consequence of the anaerobic digestion of algal biomass), photobiologically produced biohydrogen, or biodiesel, which can be obtained from algal oil. In addition microalgae contain high-added-value components such as polyunsaturated fatty acids, vitamins and pigments which play important role in food, pharmaceutical and cosmetic industries (*Guedes et al., 2011, Koberg et al., 2011*). When compared with the extensive land requirement and slow regeneration of crops and other plant tissues, the fast growth rate and specific accumulation or secretion of the substances to be recovered, convert the algal biomass into a potential competitor in the field (*Chisti 2007*). The quality and quantity of the compounds of interest can be optimized by nutritional and environmental factors, cultivation conditions and optimal harvesting at certain growth phases (*Mata et al., 2010, Guedes et al., 2011*).

A cost effective and sustainable process for obtaining bio-products from microalgae is not yet available nowadays. To achieve this target, the different process stages are under extended research. First to be mentioned are the upstream processes: species screening or genetic modification of the strains, the cultivation conditions (open ponds or closed bioreactors) and nutrition (direct caption of industrial CO_2 outlet and wastewater usage as growth medium). To continue, further challenges in the downstream scenario such as harvesting (flocculants, high throughput centrifuge) and the recovery process of the substances (dehydration for dry extraction, wet extraction, extraction condition optimization, cell disruption methods) are also under exploration in order to achieve energy efficiency and high throughput production (*Mata et al., 2010, Sorguven and Özilgen 2010, Halim et al., 2011, Pfromm et al., 2011*).

The replacement of fossil fuels has not been achieved so far by microalgae technology, where economic, environmental and consumer issues must be altogether satisfied. Several process stages must still be improved to satisfy economic criteria. Metabolic and genetic engineering is enhancing algal biology to obtain high oil content strains, while advances in photobioreactor engineering can achieve a more stable biomass production. Finally,

downstream processes (harvesting and oil extraction) have to be intensified in order to reach a sustainable biodiesel process (*Chisti, 2007, Huang et al., 2010).*

Different cell disruption methods can be used to enhance the extraction of valuable intracellular components from microalgae. Among various mechanical, chemical, thermal methods, and other novel methods (*Lee et al., 2010, Halim et al., 2011, Wiltshire et al., 2000, Halim et al., 2012, McMillan et al., 2013, Šoštarič et al. 2012*), microwave treatment has been proposed for cell wall rupture. As an oscillating electric field, microwaves cause rapid alignment and realignment of dipoles in a polar solvent, which produces rapid heating. Internal vapour generation in materials with high water content establishes a pressure gradient inside the sample (*Lyons and Hatcher 1972, Navarrete et al., 2011*). Such a pressure difference may cause the damage of the algal cell walls, and let the intracellular material become more accessible to the solvent.

Microwaves have been applied as a pre-treatment prior to solvent extraction, thus only the biomass is involved in the irradiation stage, in the absence of the organic solvent (*Lee et al., 2010, Balasubramanian et al., 2011, Biller et al., 2013*). In other studies (*Pasquet et al., 2011, Iqbal and Theegala, 2013*) microwave assisted extraction (MAE) was performed to extract algal pigments or lipids, where the solvent and solute were both exposed together to the microwaves. In the case of the pigment extraction process, the non-polar hexane solvent did not react to the microwave irradiation, but immediately dissolved the released intracellular components (*Pasquet et al., 2011*). For lipid extraction, it was found that biodiesel can be used as co-solvent in MAE as an alternative to the toxic hexane (*Iqbal and Theegala, 2013*). Other organic solvents, like alcohols, react better to microwaves, thus allowing microwave assisted transesterification in the presence of catalysts, with methanol as a reactant (*Barnard et al., 2007*). By connecting these two features, a combined one-step extraction – conversion technique can also be used for biodiesel production (*Patil et al., 2011, Patil et al., 2012*).

There are plenty of problems to be solved in order to achieve a sustainable production of biofuels or other valuable substances from microalgae. Among these, the cell disintegration step will always play an important role, since cells need to be disrupted to release intracellular lipids from microalgae. However, the achievement of an energy efficient method has proven to be difficult to overcome, but necessary to facilitate and

upgrade the extraction process, by reducing the requirement of solvent stock while still obtaining high oil and high-value products recoveries (*Chisti, 2007, Mata et al., 2010*). The selected technique must be adaptable to the likelihood of a variable algal feedstock.

The aim of this study was to investigate the effect of microwave pre-treatments on extraction kinetics from different microalgae species using different extraction processes. The object, in this case, was that whether the algal specie, the obtained product, or the extraction technique were different, it would always be possible to demonstrate the cell damage due to the microwave irradiation. Microwave energy was applied on concentrated microalgae paste to achieve the heating up of the intracellular water. Extraction kinetics measured in Soxhlet extraction from dried *Nannochloropsis gaditana* and in stirred extraction from wet *Secenedesmus almeriensis* were studied. Gravimetric analyses, UV-Vis spectrophotometry, scanning electron microscopy and fatty acid composition determination by gas chromatography were performed in order to follow the changes produced by the microwave pre-treatments on the algal biomass.

2. MATERIALS AND METHODS

2.1. Microalgae

Two different microalgae species were used in the experiments. Harvested and dewatered *Nannochloropsis gaditana* (for lipid extraction) samples were received from the Department of Vegetal Production (University School of Agricultural Engineers, Universidad Politécnica de Madrid). Initial water content was 84%. Samples were treated with microwaves within 2 days of their reception, dried and stored under vacuum at 4ºC until extraction.

The other species, *Scenedesmus almeriensis* (principally for pigment extraction) was received from "Estación Experimental Las Palmerillas", Almeria, Spain. Initial water content was approximately 90%. Some samples were treated and extracted on the arrival date, the others were kept in the freezer and later defrosted prior to the microwave treatment, followed by extraction.

As microalgae deteriorate rapidly after harvesting, only a few experiments were performed with fresh microalgae. The rest of the raw material was frozen to avoid loss of

active compounds. To ensure that storage of samples does not alter the extraction behaviour of the raw material, some conventional extraction experiments were performed with fresh and defrosted algae, and results were compared.

2.2. Moisture content determination

The moisture content of the microalgae was determined before and after microwave treatments. Samples were dried at 105°C for 24 hours. Moisture content was calculated from the wet and dry masses.

2.3. Experimental scheme

Experiments were carried out in two series, referred to as "Experiment I" and "Experiment II" (Figure 1). In both experimental series, the microwave treatment was carried out on centrifuged microalgae paste in order to take advantage of the microwave energy for the heating of the intracellular water. In "Experiment I" *Nannochloropsis gaditana* was used for lipid extraction. The microwave pre-treatment was carried out in a domestic microwave oven. Next, samples were dried at 60 °C for further extraction. Hexane was used as the extraction solvent in a Soxhlet apparatus, where samples were taken from the boiling liquid in order to measure the kinetics of the extraction. One sample (the Control) did not suffer microwave pre-treatment, but followed the same drying and extraction process in order to compare the effect of the pre-treatment. Extracts were analysed using gravimetry and spectrophotometry. Cell surface changes in microalgae were observed using scanning electron microscopy.

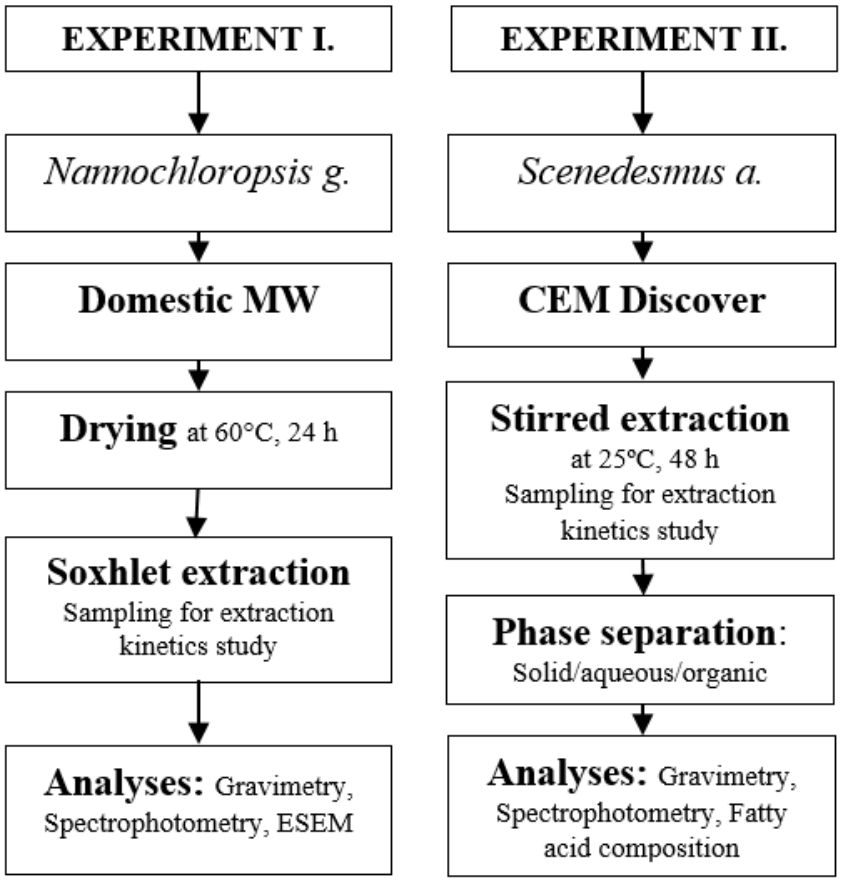

Figure 1 *Experimental procedures for "Experiment I" and "Experiment II".*

In "Experiment II" *Scenedesmus almeriensis* was used for pigment extraction. The microwave pre-treatment was performed in a laboratory microwave device (CEM Discover ONE). The wet biomass was extracted with hexane in continuously stirred extraction vessels at 25ºC. In continuation, solid, aqueous and organic phases were separated by centrifugation. The organic phase was analysed by gravimetry and spectrophotometry, and fatty acid composition was determined by gas chromatography.

Detailed conditions of the experimental steps are presented below.

2.3.1. Microwave treatments

2.3.1.1. Domestic microwave oven

"Experiment I" was performed in a modified domestic microwave oven (Figure 2) (Panasonic NNGD-566M; oven cavity: 359 x 352 x 217 mm) with a maximum output power of 1000 W. In order to promote cell wall rupture, experiments were performed at ambient pressure. In each experiment, 60 g of wet algae paste was divided into three polycarbonate tubes (Nalgene) with a diameter of 38 mm. The tubes were connected to a general collector, allowing the produced vapour produced to be condensed as it exited the system. After exposing the microalgae to microwave radiation, evaporated and condensed water were weighed and refilled into the tubes to maintain the TS (total solid) content in the samples. Temperature was recorded before and after MW pre-treatment. Experiments were carried out an emitted energy level of at 0.5 - 1.0 - 1.5 *kJ/ml* to obtain maximum treatment temperatures of 65, 80 and 88 °C, respectively.

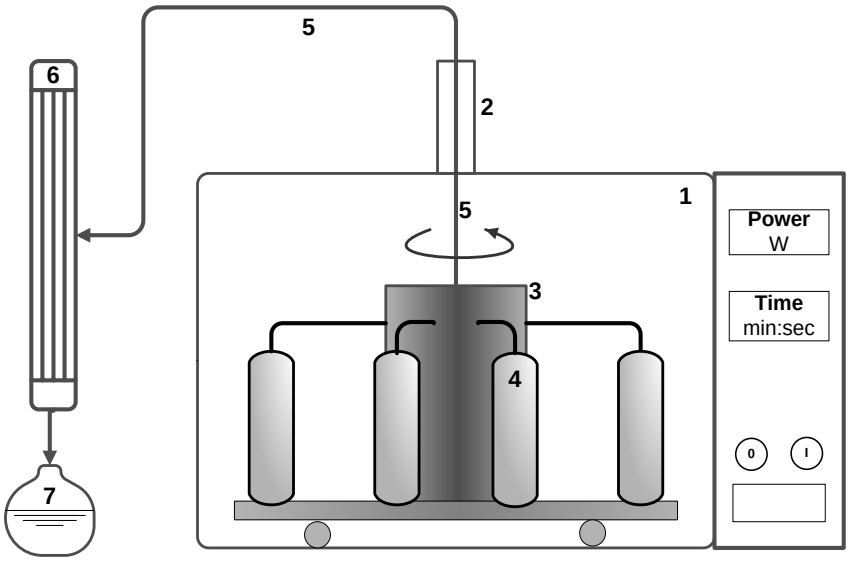

Figure 2 *Domestic microwave oven used in "Experiment I": 1 – microwave oven; 2 – tube welded onto microwave cavity; 3 – turning carousel with vapor collection (r = 160 mm); 4 – Polycarbonate tubes (85 ml, d= 38 mm); 5 – silicon tube for vapor exit; 6 – condenser*

2.3.1.2. CEM Discovery laboratory equipment

"Experiment II" was carried out in a laboratory microwave equipment (CEM® Discover ONE) with a maximum output power of 300 W, although only 150 W was applied. 50 g of wet *Scendesmus a.* was placed in a 100 ml round-bottomed flask, open to the atmosphere. The homogeneity of microwave irradiation is questionable in any volumetric device; therefore, the algal biomass was continuously stirred with a mechanical stirrer at 50 rpm. Although the device was equipped with an infrared thermometer, the temperature inside the flask was measured with a fibre optic temperature sensor both before and after the treatment. Experiments were carried out at an emitted energy level of 0.18 - 0.24 - 0.30 *kJ/ml*, where final temperatures of 47, 68 and 86 °C were obtained, respectively.

2.3.1.3. Determination of absorbed microwave energy

The nominal emitted energy in microwave processing can be easily calculated as the product of the treatment time by the applied power. However, this emitted energy is not fully absorbed by the material. Moreover, the use of different devices or just a different geometry of the experimental setup also affects the fraction of energy which is absorbed by the sample. Since only the absorbed energy can produce an effect in the sample, it is necessary to use the absorbed instead of the emitted energy as the variable to evaluate the performance of the process. The energy absorbed by microalgae ($E_{absorbed}$ *[kJ/ml]*) has been estimated with an energy balance of three contributers (Eq. 1):

$$E_{absorbed} \approx Q_{sensible} + Q_{latent} + Q_{loss} \quad (1)$$

The sensible heat contribution ($Q_{sensible}$) was calculated from the increment in temperature before and after microwave treatment, and the latent heat contribution (Q_{latent}) from the weight of evaporated water. The heat loss from the vessel surface (Q_{loss}) was evaluated through the heat transfer coefficient of the system. This coefficient was obtained by correlating the decrease in temperature over time of a hot sample placed into the microwave cavity without microwave irradiation. Detailed determination of the absorbed energy has been explained elsewhere (*Sólyom et al., 2011*). In the case of the laboratory equipment (Experiment II) this heat loss from the vessel surface was negligible due to the lack of free space inside the cavity.

The relation between the absorbed and emitted energy gives the efficiency of the experimental device (Table 1).

Table 1 *Calculation of Microwave Absorption Efficiency and Emitted and Absorbed energies from experimental final heating temperature and evaporated water, in domestic and laboratory microwave setups.*

	Temperature	Evaporated water	E emitted	E absrobred	Efficiency
	°C	wt%	kJ/ml	kJ/ml	%
domestic MW	65	0.17	0.5	0.22	44
	80	1.83	1.0	0.41	41
	88	5.83	1.5	0.78	52
CEM® Discover	47	0.5	0.180	0.151	84
	68	0.4	0.240	0.234	97
	86	0.0	0.300	0.297	99

2.3.2. Extraction methods

2.3.2.1. Soxhlet extraction

Soxhlet extraction was performed with n-hexane in "Experiment I" to obtain the lipid fraction from *Nannochloropsis g*. This method was chosen because it has been frequently used as a reference technique in lipid extraction (*Balasubramaniam et al., 2011, Halim et al., 2011*). Microalgae provided by the microwave treatment was dried at 60ºC for 24 h prior to solvent extraction, in order to avoid immiscibility problems because of polarity difference between solvents. A Soxhlet extractor (250 ml) was used with a ratio of 60 ml hexane/gram of dry material, and 6.5 reflux/h for 4 hours. Samples were taken from the boiling flask at different extraction times, using a 1 ml syringe, in order to follow the extraction kinetics in real time.

2.3.2.2. Continuous stirred extraction

Scendesmus a. was extracted directly after the microwave treatment in its wet form. Despite the very slow mass transfer between the aqueous and organic phase, this technique may be interesting on an industrial scale as the high cost of drying, prior to the extraction, can be saved. Nevertheless, the extraction process may be enhanced to achieve better contact between the two phases; in this study, it was found to be sufficient for the evaluation of the microwave pre-treatment effect. Extraction was performed at 25 °C in a 100 ml closed bottle, without previous drying. 60 ml of hexane was applied to 5 g wet

biomass, and continuously stirred using an orbital shaker at 350 rpm. Centrifuge was used for phase separation (10 000 rpm, 10 min) after the extraction, and the organic phase was further analysed.

Two different sources of *Scendesmus* were used:

1) Fresh microalgae. It was first pretreated (0.18 - 0.24 - 0.30 *kJ/ml* emitted energy level) and then extracted in duplicates. Also a control sample, without microwave pre-treatment, was used for extraction. Solvent samples of 1 ml were taken from the organic phase over 24 hours, and analysed by spectrophotometry. Only the final extract was analysed by gravimetry.

2) Frozen microalgae. It was defrosted and pretreated with microwave at 0.30 kJ/ml emitted energy level. Duplicates with control (defrosted, untreated sample) were extracted over 6-12-24 hours, and all of them were analysed by gravimetry and spectrophotometry. The fatty acid composition was determined from the accumulated extract.

2.3.3. Analytical procedures

2.3.3.1. Gravimetric analysis

The extracted material was quantified after solvent evaporation (Büchi Rotavapor, 40 °C, 50 rpm). The dried extract was later re-dissolved in hexane and used for spectrophotometry calibration.

2.3.3.2. Spectrophotometry

In the case of "Experiment I", the extracts were measured at the maximum absorption wavelength (498 nm) to follow colour evolution during extraction (Hitachi U-2000). In the series of "Experiment II", a wavelength scan was performed (Shimadzu UV-Vis Spectrophotometer) between 200 and 800 nm to detect peaks of different pigments.

2.3.3.3. Environmental Scanning electron microscope (ESEM) analysis

The effect caused by microwave pre-treatment on extraction kinetics gives an indirect response of the cell disruption produced by microwaves. In addition, Environmental Scanning Electron Microscopy (ESEM-Quanta 200-F) of untreated algae and algae exposed to microwaves was used to obtain a qualitative impression of this phenomenon in "Experiment I".

2.3.3.4. Fatty acid composition - Gas chromatography

Fatty acids contained in the cumulative extract from the control and pre-treated *Scendesmus a*. samples were analysed in Experiment II by the *CSIC Instituto de la Grasa, Sevilla (Spain).* Component identification was performed by monitoring retention time coincidence with the following standard components: C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (linolenic acid), C18:4, C20:0 (arachidonic acid), C20:1 (eicosenic acid), C20:2 (n-6), C20:4 (n-6), C20:5 (n-3) (EPA).

3. RESULTS AND DISCUSSION

3.1. Experiment I - Nannochloropsis gaditana

3.1.1. Extraction kinetics

The extraction kinetic curves shown in Figure 3 were obtained from absorbance measurements, correlated to the gravimetric analysis. Extraction yield was expressed in terms of g extract/g dry matter. The evolution of the accumulated yield *(Y)* can be described by first order kinetics to quantitatively compare different extractions (Eq.2).

$$Y = Y_{max}[1 - exp(-\beta t)] \quad (2)$$

In Equation (2), extraction kinetics is described by two parameters: the pre-exponential parameter (Y_{max}), which represents the maximum yield reached at infinite extraction time, and the initial extraction rate $\left(\left.\frac{dY}{dt}\right|_{t=0}\right)$, which is calculated as the product of the maximum

yield and the time constant (β [min^{-1}]). First order kinetic parameters were obtained by a regression fit, minimizing the quadratic difference of the experimental ($Y_{experimental}$) and calculated ($Y_{calculated}$) extraction yields as the objective function *(O.F.)* with Microsoft® Office Solver (Eq. 3)

$$O.F. = \Sigma\,[(Y_{experimental} - Y_{calculated})^2] \quad (3)$$

Calculated parameters of first order kinetics and experimental gravimetric yields, listed in Table 2, show a considerable increment (83-103 %) in extraction yields and also an important increment of initial extraction rates after every microwave pre-treatment. Moreover, these increments do not depend on the amount of energy used in the pretreatment. Although there is some discrepancy between correlated and gravimetric values, it is almost negligible at the higher energy levels.

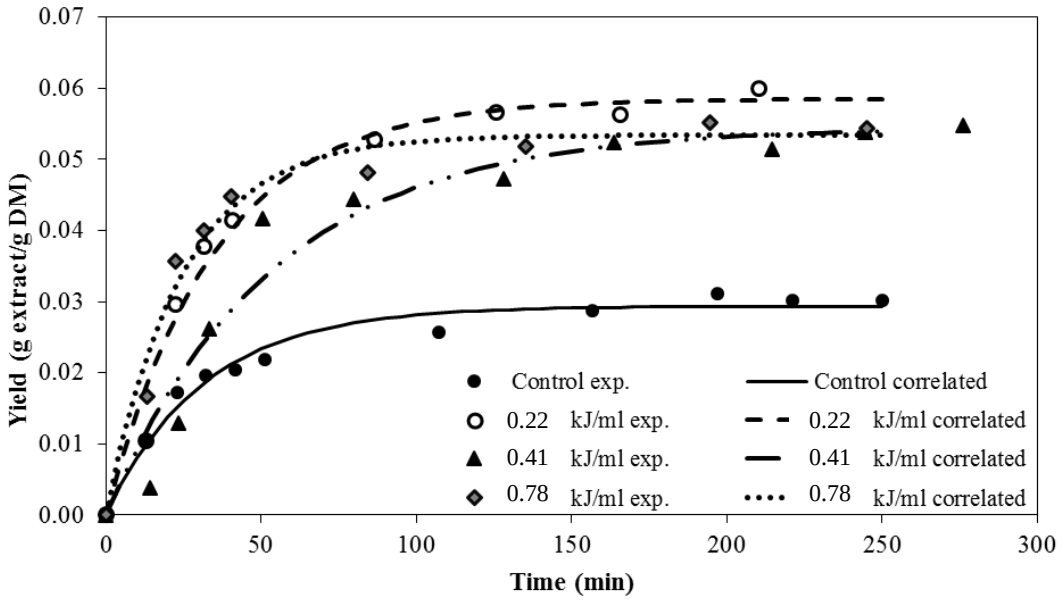

Figure 3 *Correlated and experimental kinetic curves of lipids extraction from Nannochloropsis gaditana, obtained by spectrophotometry.*

The results presented here are not compound specific and further analysis should be carried out for a more detailed knowledge. However, it is here demonstrated here that microwave pre-treatment on fresh wet material, without added organic solvent, is able to shorten the extraction process. Similar tendencies were observed in other studies on diluted microalgae (*Balasubramanian et al., 2011*) and microwave pre-treatment was found to be the second most effective one (after ultrasound) when compared with, water bath, blender and ultrasound prereatments (*McMillan et al., 2013*).

Table 2 *First order kinetic parameters and gravimetric yields of lipid extraction at different absorbed energy levels from Nannochloropsis gaditana*

$E_{absorbed}$	$Y_{max\ gravimetric}$	$Y_{max\ correlated\ *}$	**Initial extraction rate**:** $Y_{max} * \beta$
kJ/ml	g/g dry matter	g/g dry matter	g/g DM*min^{-1}
Control	0.037	0.029	$9.31 * 10^{-4}$
0.22	0.077	0.059	$1.68 * 10^{-3}$
0.41	0.052	0.054	$1.02 * 10^{-3}$
0.78	0.048	0.053	$2.19 * 10^{-3}$

**Y_{max} 11.9 % error from duplicate*

***β 26.1 % error from duplicate*

3.1.2. ESEM analysis

Qualitative analysis was made by environmental scanning electron microscope to compare the changes on the cell wall after microwave pre-reatment. On the "Control" sample (Figure 4 a) a smooth cell surface can be observed, while the cell surface of the algae which suffered MW treatment (Figure 4 b) became creased and contracted. An evidence of cell explosion cannot be clearly seen on the figures, nevertheless a defined damage occurred due to the MW irradiation.

a) b)

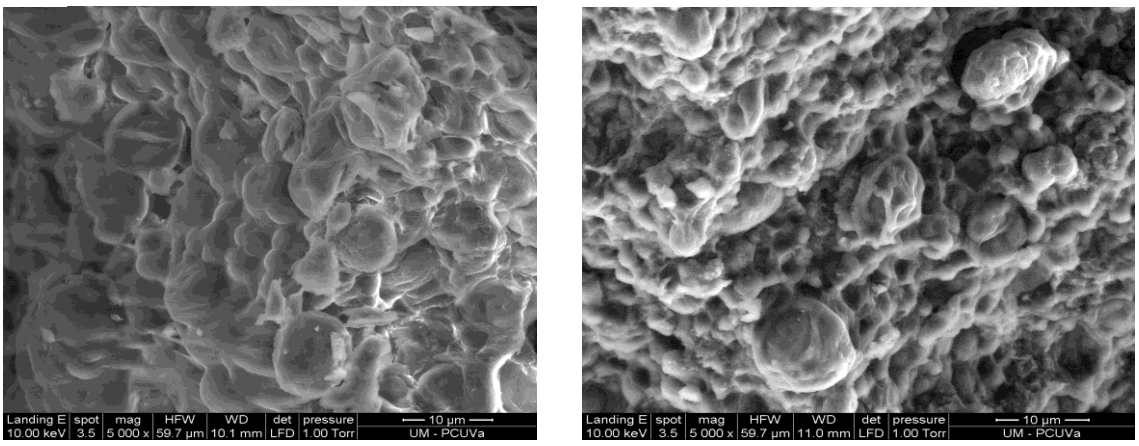

Figure 4 . *ESEM picture of Nannochloropsis gaditana: a) before and b) after microwave pre-treatment*

A semi-quantitative cell diameter evaluation of microscopic pictures showed higher average diameter for the "Control" sample (145 cells were measured), where 42% of the counted cells were above the average value (≈ 7 µm). When measuring the cells after the

microwave treatment (190 cells were measured), the average diameter was lower than 6 μm, and only the 25% were larger than the average size of "Control". The disappearance of the larger cells may suggest their vulnerability to the pre-treatment, although the disintegrated cell debris and cell wall residues cannot be separately observed by the microscope. SEM pictures from microwave assisted extraction (*Iqbal and Theegala, 2013*) showed similar damage on the cell surface, although there the solvent was also involved in the microwave treatment.

3.2. Experiment II – Scenedesmus almeriensis

3.2.1. Extraction kinetics

In the first part of "Experiment II", fresh microalgae was exposed to MW irradiation, then cooled down, and further extracted by stirring with hexane at 25ºC, without the intermediate drying step used in "Experiment I". Again, a control sample, without microwave pre-treatment, was also processed. The evolution of the organic phase was studied using spectrophotometric wavelength scan between 200 and 800 nm. Peaks of pigment substances were identified in all experiments at 669±1, 471±2, 442±2 and 416±2 nm. The first wavelength corresponds to chlorophylls and the other three peaks group together a mixture of carotenoid and chlorophyll compounds. As the peaks are overlapping in the visual light range, for separate analysis of the pigments more specific analytical techniques would be required, such as high performance liquid chromatography (*Minguez Mosquera et al., 1992, Zang et al., 1997, Cerón et al., 2008*) or thin layer chromatography (*Lichtentaler and Buschman, 2001*). However, the spectrophotometric results obtained here have proved to be good enough to provide the quantitative content of pigment compounds which was required for the aim of this paper. Also, the use of accurate extinction coefficients at different wavelengths could help to improve the precision of the results. Nevertheless, there is information available in literature (*Lichtentaler and Buschman, 2001, Cvetković and Marković 2008*) for several components in different solvents, the usage of these coefficients is considered misleading in this study due to the complexity of the natural extract mixture from microalgae. To obtain a general view of the progress in the extraction, this study evaluated the absorbance value at 442±2 nm. In all the experiments similar amounts of solvent and wet microalgae were used. However, the concentrations of the extracted samples in kinetic analysis were

close to each other. For more precise determination, the absorbance values were evaluated based on the dry microalgae weight found in the extraction vessel. The measured peak probably corresponds to the amount of lutein in the extract mixture, which can be gained from *Scenedesmus almeriensis* and is of interest to the food industry as a colorant (*Cerón et al., 2008*).

A significant increment can be observed in the final absorbance values after 24 hours of extraction (Figure 5). The pre-treatment at 86 °C resulted in 11.3 $A_{440\,nm}/g_{\,dry\,matter}$, a 161% relative increase over the Control sample. In the cases of pre-treatments at 68 and 47 °C, absorbance values of 5.80 and 5.60 $A_{440nm}/g_{\,dry\,matter}$ were achieved respectively, while the control sample resulted in 4.21 $A_{440\,nm}/g_{\,dry\,matter}$ (relative increments of 38% and 33%, respectively). The magnitude of increment is comparable with the results obtained in "Experiment I" despite the different experimental conditions. Literature on pigment extraction from microalgae using microwaves is scarce (*Pasquet et al., 2011*); however, when microwave is compared to other techniques, it is found to be effective, rapid, and also causes no thermal degradation of the compounds (*Cravotto et al., 2008, Patil et al., 2011*).

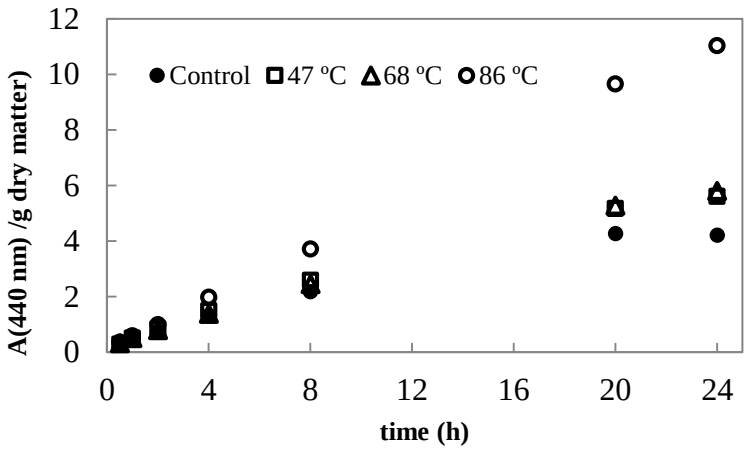

Figure 5 *Experimental results of extraction kinetics of pigments from Scenedesmus almeriensis at different temperatures, compared to untreated algae (Control).*

In the second part of "Experiment II", the same microwave pre-treatment as that of the first part was performed again, but only at 86 °C, in order to compare the control and pre-treated samples via spectrophotometry, gravimetry and fatty acid composition. Extractions were carried out for 6-12-24 h long in the same way as in former experiments.

The gravimetric yield of both Control and pre-treated sample were seen to increase over the time (Figure 6a), and a significant difference was not observed in terms of extract mass. Additionally, the maximum extraction yield was not achieved in 24 hours due to the slow mass transfer between the aqueous and organic phases in the present extraction procedure. By contrast, when the values of $A_{440\ nm}/g_{\ dry\ matter}$ were compared (Figure 6b), the absorbances after 24 hours were 3.75 ± 0.61 and 7.63 ± 0.68 for control and pre-treated samples respectively. Results obtained from fresh (Figure 5) and frozen (Figure 6) microalgae are comparable, indicating that the method of storage of the raw material does not appreciably alter its behaviour in the extraction process.

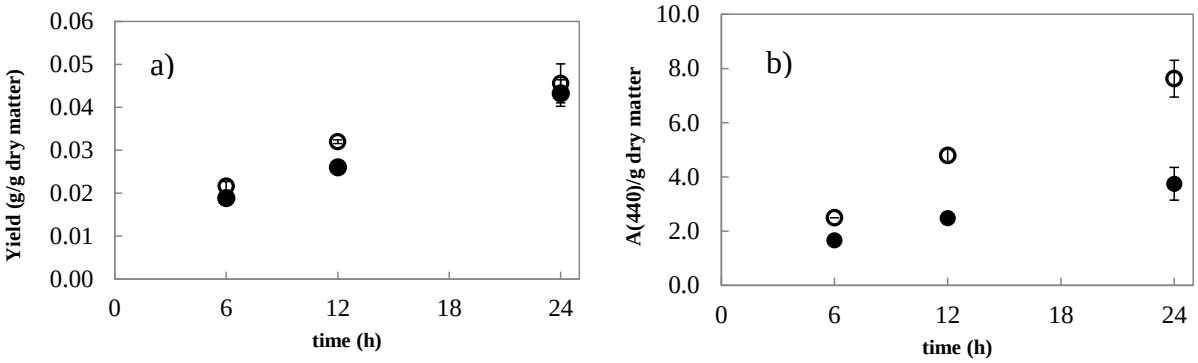

Figure 6 *Comparison between the gravimetric yield (a) and absorbance at 440 nm (b) of (●) Control and (○) MW treated (86 °C) Scenedesmus almeriensis after 6-12-24 hours extraction.*

For a better comparison between the extraction yields and spectrophotometric results, lipids and other non-colorant components should be removed from the extract. The method proposed by Cerón and co-workers (*2008*) enables the isolation of lutein from *Scenedesmus almeriensis,* and the results obtained here suggest the applicability of a microwave pre-treatment to achieve enhanced process kinetics.

3.2.2. Fatty acid composition

Accumulated extracts of "Experiment II" were analysed to determine the fatty acid composition of *Scenedesmus a*. Obtained values indicate (Table 3) that the quality of the extracted lipids after microwave pre-treatment remained roughly unchanged compared to the untreated sample, in accordance with the results of other pre-treatments (*Wiltshire et al, 2000, Cravotto et al., 2008*). Thermal degradation of the bioactive and health

beneficial compounds was avoided because of the short pre-treatment at 86°C. As the control extraction was performed at room temperature, the decrease in unsaturated fatty acids did not occur, although it was observed by others in the case of the Soxhlet extraction reference method (*Balasubramanian et al., 2011*).

Table 3 *Fatty acid composition of Scenedesmus almeriensis extracts for Control and MW pre-treated (86°C) samples.*

Fatty acid type	*Control extraction*	*Microwave pre-treatment*
Palmitic acid (C-16:0)	6.80 %	6.20 %
Palmitoleic acid (C-16:1)	2.90 %	2.40 %
Stearic acid (C-18:0)	3.90 %	4.00 %
Oleic acid (C-18:1)	10.00 %	9.60 %
Linoleic acid (C-18:2)	11.70 %	9.20 %
Linolenic acid (C-18:3)	25.30 %	30.20 %
C-18:4 n-3	2.70 %	3.00 %
Archaidic acid (C-20:0)	5.80 %	7.20 %
Eicosenic acid (C-20:1)	7.90 %	6.50 %
(C-20:2 n-6)	1.80 %	1.50 %
(C-20:4 n-6)	2.10 %	1.20 %
EPA (C-20:5 n-3)	2.30 %	2.50 %
Others	16.80 %	16.50 %
Total saturated	16.50%	17.40%
Total unsaturated	66.70%	66.10%
Total polyunsaturated	45.90%	47.60%

The percentage of total saturated fatty acids (≈17 %) and especially the amount of oleic acid (≈10 %) was low in the extracts. These substances are necessary for good biodiesel properties (*Lee et al., 2010, Halim et al., 2012*), and characteristic for other microalgae species under certain nutritional conditions. Nevertheless, the high unsaturated fatty acid content (≈66 %) and the very high polyunsaturated fatty acid amount (≈ 47 %) is

responsible for the health benefits in the nutritional usage of microalgae oil (*Balasubramanian et al., 2011*).

4. CONCLUSIONS

The positive effects of solvent free microwave pre-treatment favoured extraction of dry algae (SFMP). Although a positive influence was demonstrated by the spectrophotometric analyses, the slow mass transfer in liquid-liquid extraction of wet algae did not permit the proper study of pretreatment effects. ESEM pictures showed damaged cell surface and a reduction in the number of large cells, indicating the successful impact of the MW. Thermosensitive fatty acid compounds did not suffer degradation during the short high temperature SFMP. In general, pre-treatments enhanced the processes studied, however, their efficiency was found to be process and product dependent.

ACKNOWLEDGEMENTS

This work was supported by the Spanish Ministry of Economy and Competitiveness for the Project CTQ2010-15475 and the project of ENE2012-33613. The authors wish to thank Raul Muñoz, Javier Pereda and Miguel Ángel Álvarez for their research contribution. Katalin Sólyom thanks the University of Valladolid for the financial support provided by the "FPI UVa" scholarship.

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Chapter 3.

Dielectric properties of grape marc: Effect of temperature, moisture content and sample preparation method

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Journal of Food Engineering 119 (2013) 33-39.

DIELECTRIC PROPERTIES OF GRAPE MARC: EFFECT OF TEMPERATURE, MOISTURE CONTENT AND SAMPLE PREPARATION METHOD

Abstract

Microwave assisted extraction enhances antioxidant extraction kinetics in grape marc processing. For an effective microwave process design, the dielectric properties (DP) of the material must be known under different conditions. This study focused on the DP measurement of grape marc using a resonant cavity measuring device. Grape skin, seeds and their mixtures with three different moisture contents were studied at 28, 39 and 50 ºC. At higher moisture content DP showed increasing tendency, while temperature didn't have a definite effect. Good correlation was achieved with the experimental results applying the complex refractive index mixing equation between 0.4-0.6 porosity. Also, despite the complexity of natural products properties, the experimental DP values of the real mixture were in agreement with values estimated from DP properties of its individual constituents (grape skin and seeds). Milling was performed to obtain homogeneous material for the measurements, and this effect on the results was also studied.

1. INTRODUCTION

There is an increasing interest in antioxidant extraction from industrial by-products such as the grape marc obtained from red wine production (*Spigno and De Faveri 2007*). The conventional extraction processes can be enhanced with novel techniques such as the application of microwaves, where the dipole molecules are mostly affected by the oscillation of the dielectric field. Microwave assisted extraction processes may lead to increased extraction yields in short extraction times while using a smaller amount of solvent (*Proestos and Komaitis 2008*). However, the efficiency of any microwave process depends on the correct design of the microwave applicator. For this reason, the interaction between the treated material and the applied electric field must be known. In order to describe this interaction, the complex dielectric permittivity (Equation (1), (2)) of the material should be known (*Metaxas and Meredith 1988*).

$$\varepsilon = \varepsilon_0 \, \varepsilon_r \tag{1}$$

$$\varepsilon_r = \varepsilon_r^{'} + j\varepsilon_r^{"} \tag{2}$$

In Equation (1) ε_0 and ε_r refer to the free space permittivity and the complex dielectric constant, respectively. Equation (2) shows the real part of the complex dielectric constant ($\varepsilon_r^{'}$), which corresponds to the energy stored in the material when an electric field is applied, and the imaginary part ($\varepsilon_r^{"}$), corresponding to the energy which is converted into heat.

During the microwave process, the material undergoes physical and perhaps chemical changes (moisture content, temperature and salt concentration) which would affect its interaction with the dielectric field. These factors must be taken into account when characterizing the dielectric properties of the sample (*Venkatesh and Raghavan 2004*).

In former studies the grapes' dielectric constants were measured for microwave drying purposes (*Dev et al. 2009, Tualsidas et al. 1995*). In those measurements whole grapes or grape skin, excluding seeds, were measured in an open ended coaxial line measurement setup, requiring perfect contact between the probe and the sample. The same method was used for fresh fruit and vegetable slices, where non-monotonic dielectric behaviour was reported above 65 ºC, corresponding to the cell breakage of plant tissues (*Nigmatullin and Nelson 2006*). The dielectric properties of grape juice were studied at different frequencies (*García et al. 2001*), also with an open-ended coaxial line probe. The conductivity of the sample was also

considered, as it strongly affected the measurement results at higher frequencies. When measuring wine (*García et al. 2004*), it was reportedly difficult to establish perfect contact between the probe and the liquid sample because of the presence of disturbing CO_2 bubbles in the fermented wine. In the afore-mentioned literature all measurements were done with seedless grape berries. The inclusion of grape seeds affects the microscopic and macroscopic content of the raw material, and so dielectric properties may change. The dielectric properties of a mixture of grape seeds and skin had apparently not been measured in former studies.

When complex semi solid material is measured, open ended coaxial line probes present two main problems. On one hand, it is difficult to establish the necessary physical contact between the sample and the probe; on the other hand, the results may rely on the position and distance of the different constituent parts in the inhomogeneous material. For such samples, the resonant cavity method may be more suitable, if measurements are performed at a constant frequency (*Nelson 1991*), because this measurement method reflects the overall dielectric constant of the complex sample placed into the cavity.

Otherwise, the global dielectric properties of a multiphase material can also be estimated from the proportions and properties of their individual constituent phases by using mixing equations. Some of the previously reported correlations are derived from Maxwell's equations, while others are semi empirical models (*Sheen et al. 2010, Simpkin 2012, Nelson 2005, Erle et al. 2000*). The wide variety of equations used for different materials confirms that none of them gives the exclusive solution to the estimation of the dielectric properties of a complex mixture, and in any case, the best fit should be chosen depending on the material type, shape and complexity. Sheen et al. (*2010*) compared exponential and logarithmic models, estimated their theoretical error and correlated them with experimental data. They suggested that the complex refractive model and the random model were the most suitable for a polymer-ceramic mixture at a high volume fraction of the dispersed phase. These models are also widely mentioned in case of agricultural and food products (*Venkatesh and Raghavan 2004*). Therefore, the Complex Refractive Index Mixing model (CRIM, Equation 3), the Random model (Landau-Lifshitz-Looyenga, Equation 4) and a Logarithmic model (Lichtenecker-Rother , Equation 5) were used in this study to estimate the material dielectric properties of an air-particle mixture as a function of the sample porosity:

$$\sqrt{\varepsilon_m} = \nu_1\sqrt{\varepsilon_1} + \nu_2\sqrt{\varepsilon_2} \qquad (3)$$

$$\sqrt[3]{\varepsilon_m} = \nu_1 \sqrt[3]{\varepsilon_1} + \nu_2 \sqrt[3]{\varepsilon_2} \quad (4)$$

$$\ln \varepsilon_m = \nu_1 \ln \varepsilon_1 + \nu_2 \ln \varepsilon_2 \quad (5)$$

In Equations (3)-(5) ε_m refers to the dielectric property of the mixture, ε_1 and ε_2 correspond to the air and grape marc phases, respectively, and ν_1 and ν_2 to their volume fractions in the sample.

The object of this study was to describe the behaviour of the dielectric properties of grape marc at changing moisture content and temperature, as these are the variables which would change during a microwave pre-treatment, prior to the antioxidant extraction process. During measurement the sample holder contains a mixture of grape marc and air, as it is not possible to separate all the air from grape marc without altering the material. In order to estimate the grape marc properties themselves, it is necessary to have a proper mixing equation too. Good experimental reproducibility was obtained from homogenized material; however, homogenization could vary the measurement results. To evaluate the measured results from the homogenized samples some values obtained using the original unhomogenized material was compared to those from the homogenized grape marc. Finally, the mixing equation was applied in order to predict the dielectric properties of a grape marc mixture from the proportion and properties of their constituents: grape skin and seeds.

2. MATERIALS AND METHODS

2.1. Raw material

Fermented grape marc was provided by the Matarromera winery (Spain) in November 2011. It was a mixture of pressed, partially ruptured grape skin and whole seeds, which contributed to the colour development of the red wine during the fermentation process. For industrial reasons the samples had a 0.1 wt% of added potassium metabisulfite.

During the measurement, the initial mixture of skin and seeds was used either in milled form, or was manually separated into skin and seed fractions and measured either in milled or in original form.

Samples were stored in a freezer at -20 ºC and thawed for use in experiments at room temperature overnight.

2.2 Analysis of moisture content

Moisture content was determined by an infrared moisture analyser (Kern®-MLS) at 105 ºC until reaching a stable weight. Original moisture content was 59.7% for the milled skin and seeds mixture, which contained 42 wt% seeds and 58 wt% skin. The original seeds and skin had a moisture content of 49.2% and 65.9%, respectively.

2.3. Preparation method

Some of the samples were milled, before thawing, in a Thermomix for 20 s/100 g to obtain <1 mm particles.

Desired moisture content was achieved by drying at 40 ºC (VWR® Incu-Line IL23) at atmospheric pressure.

2.4. Density measurement

Sample density was determined for the milled and original samples, and for all moisture contents in a gas pycnometer (Quanta Chrome®), by applying the gas at room temperature. The average value of 5-fold measurements was used in further calculations.

2.5. Measurement of dielectric properties

A Püschner Dielectric Measurement Kit® was used for the measurements, with 1 ml glass vials of 35 mm height and 6.2 mm inner diameter. The device measures the frequency response (2.45 GHz) with the cavity perturbation method, where firstly the empty sample holder was measured, followed by the filled sample holder. From the frequency shift and quality factor difference, the software provided (Microwave Dielectric Kit 3.0.3®) was able to calculate the dielectric constant and dielectric loss.

For every measurement point, 6 sample holders were prepared and the dielectric properties were measured. The bulk densities (ρ_{bulk}; [g/cm^3]) were determined from the sample weight and the volume in every sample holder. Porosity (ϑ) was calculated from the density of the material ($\rho_{material}$; [g/cm^3]), and the prepared bulk density in the sample holder. (Equation 6) The average results of the dielectric properties from the 6-fold measurements are presented with the corresponding standard deviation for every measurement point.

$$\vartheta = 1 - \frac{\rho_{bulk}}{\rho_{material}} \quad (6)$$

To validate the accuracy of the measurement device, dielectric properties of distilled water were also determined in the temperature range 28-50 ºC, with deviations from literature values lower than 5 %.

Measurements at different temperatures were made by placing the measurement device in an incubation oven (VWR® Incu-Line IL23).

2.6. Conditions at which the dielectric properties were measured

For the best estimation of the dielectric properties of the pure material, measurements were made first with milled mixed grape marc at its fresh moisture content. Measurements were performed at room temperature and at different porosities (0.1-0.6). Below and above this porosity range it was not possible to maintain homogeneity along the sample holder, and no reliable results were obtained. Mixing equations were fitted to the measured data, and the most successful one was chosen for further calculations. In order to avoid the effect of porosity on experimental values, the latter were always normalized with the selected mixing equation to a porosity of 0.5. This approach allows us to compare results from different samples, regardless of their porosity.

Next, moisture and temperature dependence were measured for milled mixed grape marc, milled skin and milled seeds. Temperatures of 28-39-50 ºC were chosen, as they covered the range where the equipment gave reliable results. Three different moisture contents were used: 1) original moisture (M_1 [%]) of the samples, 2) M_2=M_1-9 % and 3) M_3=M_1-18 %.

The effect of sample preparation (milling) on the measurements was determined by comparing unmilled and milled samples, in the case of grape skin and seeds. Measurements were carried out at the original moisture content and also with dried samples, around 10% of moisture, when only bound water was assumed to be present in the material (*Navarrete et al., 2011*)

2.7. Experimental data analysis

The experimental data was analysed with two-way ANOVA with OriginLab® software. Full factorial design was applied (3^2), where the factors were the temperature and the moisture content, each at 3 levels. The different types of material (seed, skin, mixture) were considered

in separated experimental designs. Results were considered different at a significance level of $P < 0.05$

2.8. Data correlation

Dielectric properties were correlated using the following objective function ($O.F.$), which represents the percentage standard deviation of the dielectric constant and the dielectric loss (Equation 7):

$$O.F. = \sqrt{\frac{\Sigma\left(\left(\frac{\varepsilon'_{exp}-\varepsilon'_{cal}}{\varepsilon'_{exp}}\right)^2+\left(\frac{\varepsilon''_{exp}-\varepsilon''_{cal}}{\varepsilon''_{exp}}\right)^2\right)}{2N-1}} \quad (7)$$

The correlation parameters were the dielectric constant and the dielectric loss of the grape marc phase. All the three models, CRIM, Random and Lichtenecker-Rother, were used in the correlation, which was performed with Microsoft Excel Solver.

3. RESULTS AND DISCUSSION

3.1. Mixing equations for "air – particle" mixtures: effect of porosity

When comparing measured data with values calculated using the mixing equations, a perfect fitting on the whole porosity range was not obtained with any of the used equations used (Figure 1). The Lichtenecker-Rother equation fits better than the others at low porosity, but the extrapolated pure material estimation (Table 1) leads to very high values (ε'= 58.44, ε" = 16.38) which are not in a reasonable range. Moreover, it vigorously underestimates the measured values with increasing porosity. The Lichtenecker-Rother equation is considered to be a semi-empirical model, a mathematical rather than physical approach, and it has been widely used for all kinds of mixtures (Erle et al. 2000). Even so, Simpkin (*2012*) provided it with an electromagnetic justification derived from Maxwell's equations. Pereira and coworkers (*2002*) found this equation less suitable than other models for an ethanol-polymer mixture at different temperatures. They suggested the complex refractive index model (CRIM) for their mixture, which is in accordance with Nelson (2005) in the case of cereal grains.

Table 1 *Dielectric properties of zero porosity grape marc and objective function (O.F) from data regression of milled grape marc as a function of porosity.*

Model	$\varepsilon'_{v=0}$	$\varepsilon''_{v=0}$	*O.F.*
CRIM	39.23	6.73	16.0%
Random	44.45	8.65	18.1%
Logarithmic	58.44	16.38	36.3%

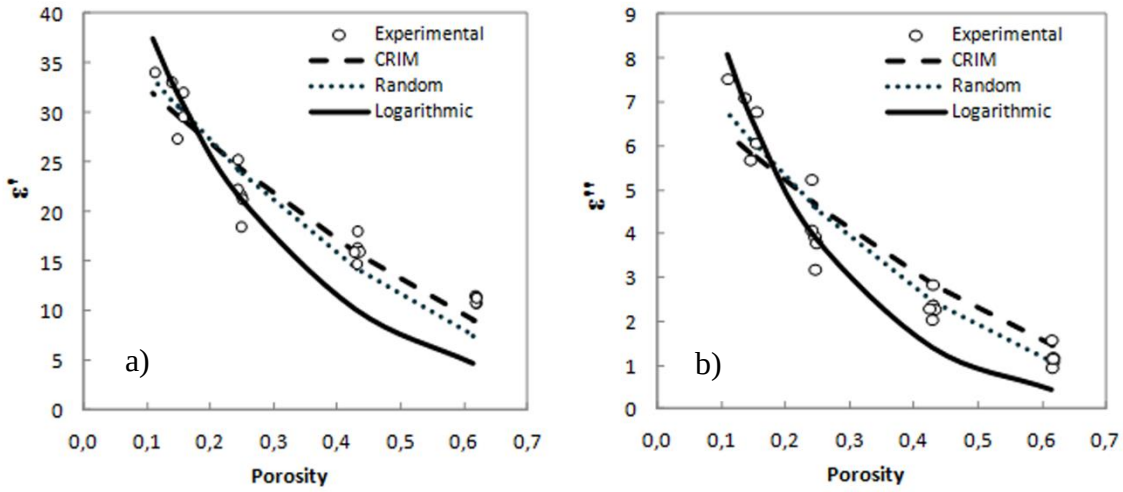

Figure 1 *Dielectric constant (a) and dielectric loss (b) data regression for milled grape marc (seeds and skin mixture)*

The fits of the complex refractive index model and the random model are very close to each other, and give good correlation with the measured values in the porosity range of 0.4-0.6. Also, in this range, the results gave the best repeatability since the original porosity of the sample remained unmodified. On the other hand, these equations could be not accurate when extending the porosity range to higher and lower values, where experimental reproducibility was lower. These two equations were found suitable for mixtures of different types of materials. (*Venkatesh and Raghavan 2004, Sheen et al. 2010, Nelson 2005, Pereira et al. 2002*)

In order to allow comparisons to be made in the following sections between values measured under different conditions, regardless of their actual porosity in the experimental procedure, it was considered convenient to convert the experimental values to a fixed porosity condition. These adapted values would be obtained from the experimental ones, considering porosity influence by means of a mixing equation. As the correlation with CRIM gave the lowest value of the minimization objective function in the porosity range from 0.4-0.6, it was chosen in the

present study, in order to further convert experimental measurement results to a fixed porosity condition. The chosen fixed porosity value was 0.5, since this value is close to the experimental ones, and the mixing equation behaves properly in this region.

3.2. Comparison of whole and milled products: effect of sample preparation

In order to improve repeatability, the product was homogenized prior to measurement. However, former studies mentioned the possibility of changing the measured results by applying of mechanical forces for crushing, or changing the sample's size and shape before the measurement. (*Kent and Kress-Rogers 1986, Venkatesh and Raghavan 2004*)

Table 2 *Comparison between whole and milled samples at 28 ºC*

	Moisture	Experimental			CRIM			Linear Model (Eq. 8)		
	%	Porosity	ε'	ε''	Porosity	ε'	ε''	Porosity	ε'	ε''
Whole seeds	46.7	0.5247 ± 0.0307	6.06 ± 0.23	0.771 ± 0.053	0.5	6.27 ± 0.20	0.787 ± 0.031	0.5	-	-
	10.6	0.6441 ± 0.0076	2.53 ± 0.09	0.171 ± 0.021	0.5	3.38 ± 0.18	0.281 ± 0.041	0.5	-	-
Milled seeds	49.2	0.4990 ± 0.0020	8.73 ± 0.14	1.644 ± 0.036	0.5	8.71 ± 0.16	1.639 ± 0.035	0.5	-	-
	11.8	0.5593 ± 0.0007	2.91 ± 0.03	0.215 ± 0.061	0.5	3.24 ± 0.04	0.258 ± 0.008	0.5	-	-
Whole skin	64.4	0.5440 ± 0.0014	11.44 ± 1.31	1.878 ± 0.126	0.5	12.63 ± 0.63	2.131 ± 0.140	0.5	-	-
	8.6	0.8877 ± 0.0077	1.93 ± 0.05	0.055 ± 0.006	0.5	7.59 ± 0.44	0.488 ± 0.050	0.5	1.45 ± 0.02	0.027 ± 0.002
Milled skin	65.9	0.4789 ± 0.0008	11.78 ± 0.40	1.985 ± 0.101	0.5	10.83 ± 0.15	1.778 ± 0.032	0.5	-	-
	10.0	0.6704 ± 0.0013	2.53 ± 0.02	0.129 ± 0.004	0.5	3.59 ± 0.03	0.233 ± 0.005	0.5	1.76 ± 0.09	0.064 ± 0.002

When grape seeds and grape skin were compared at their original moisture content in whole and milled form, some differences were observed in the results (Table 2). In the case of the milled fresh seeds a higher dielectric constant was obtained which could be explained by the higher moisture content of the sample. When the same material was compared after drying (a moisture content close to 10%, which corresponds to the bound water in the matrix) a significant difference was not observed between the values of the dielectric constant and the loss in both samples, whole and milled grape seeds. This corresponds with Navarrete et al. (*2011*) who also found that the contribution of bound water to the dielectric properties is negligible compared to the contribution of free water in high moisture samples. The values measured with the unmilled samples showed high experimental error, which also justifies the need for the milling step.

In the case of the fresh grape skin, when converted values to 0.5 porosity are compared, both a higher dielectric constant and loss were observed for the whole skin (ε' = 12.63, ε" = 2.131) than for the milled product (ε' = 10.83, ε" = 1.778). However, the opposite effect was expected because of the higher moisture content of the milled skin.

This effect was even more pronounced with the dry material, which is again in contrast to the expectations. In experiments with dry skin the porosity of the sample (0.8877 and 0.6704) was considerably higher than 0.5 due to the fragility of the dry material (Table 2). When these values were adapted to 0.5 porosity with the CRIM model, no conclusive results were obtained. This behaviour may be explained by the reported lack of suitability of the CRIM equation for high porosity mixtures. (*Sheen et al. 2010*) In this range, above 0.7 porosity, the same authors proposed the use of a linear approximation (Equation 8)

$$\varepsilon_m = v_1\varepsilon_1 + v_2\varepsilon_2 \quad (8)$$

The experimental values of dry grape skin were adapted to 0.5 porosity also using the Linear model (Table 2). With this model, a slight but significant increment of dielectric constant can be observed for milled skin ($\varepsilon' = 1.76$) when compared to whole skin ($\varepsilon' = 1.45$). In the case of dielectric loss the value was almost doubled ($\varepsilon" = 0.064$ for milled, and $\varepsilon" = 0.027$ for whole skin). Obviously, it is hard to obtain reliable results in these conditions and extrapolate them to the pure material or to a porosity of 0.5. Besides porosity, differences in sample shape could also explain the discrepancies between the results. (*Jones and Friedman 2000, Di Biasio and Cametti. 2007*.) Despite the non-equivalent results, it was expected that a similar tendency in dielectric property behaviour at different conditions of moisture content and temperature would be obtained through further experimentation (Section 3.3).

3.3. The effect of moisture and temperature on the milled mixture, skin and seeds

A clear influence of the moisture content on the dielectric constant and dielectric loss was noted, such that higher moistures lead to higher dielectric constant and loss for all types of studied materials at any temperature (Figure 2). As all studied materials had relatively high moisture content, the observed phenomena was in accordance with the rule: the more free water is in the material, the higher the microwave absorption and energy dissipation will be (*Metaxas and Meredtih 1988*).

There was not such clear evidence regarding the effect of temperature. In the case of the dielectric constant, the values were seen to increase slightly with increasing temperature at every moisture content. However, a clear decrease in this property has been described in literature (*Dev et al. 2009, Tulasidas et al. 1995*), as happens with water. This discrepancy in behaviour may be explained by the presence of potassium metabisulfite (0.1 wt %) added to the raw material during the industrial processing of grape marc. Moreover, the increasing value of dielectric properties with temperature at high moisture content may also be explained by the complex water – oligosaccharide secondary bonds in the natural matrix. Sipahioglu and Barringer (*2003*) found similar behaviour for garlic samples with 57.3 % moisture content, where the increasing tendency was due to the high inulin content of the samples.

In the case of the dielectric loss of the milled seeds at the highest moisture content, a slight decrease in behaviour was observed when temperature was increased, as is expected when a high amount of free water is present. This may be explained by the lower salt content in the seeds, as the whole seed is a closed structure, in which salt is only found on the outer layer of the seed. The inner part of the seeds remains intact due to the protective function guarding the possible future reproduction of the grape plant. At lower moisture content, the dielectric loss increased with temperature.

Although the former interpretation of the results is hypothetical, neither the clear water-like behaviour nor the obvious salt effects were observed through the experimental data.

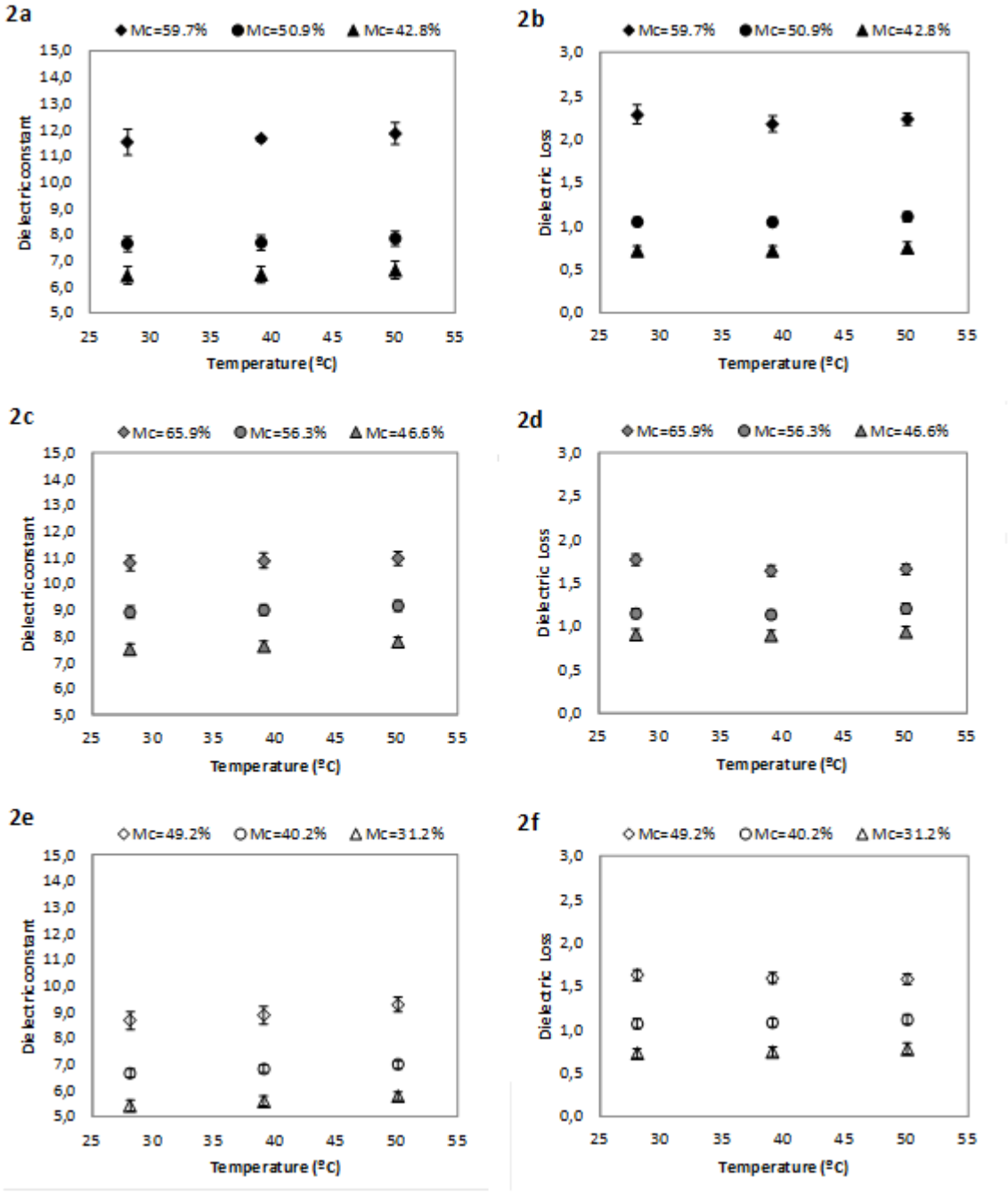

Figure 2 *Dielectric constant (a) and dielectric loss (b) of milled grape marc (seeds and skin mixture), dielectric constant (c) and dielectric loss (d) of milled skin, dielectric constant € and dielectric loss (f) of milled seeds at v= 0.5.*

All these results were fitted with a quadratic regression model (Equation 9) to determine the complex dielectric properties at any moisture content (Mc [%]) and temperature (T [ºC]) in the measured range. The general model takes into account the linear, quadratic and crossing dependence on moisture and temperature through the corresponding coefficients for temperature (k_T), second order temperature effect (k_{T^2}), joint temperature and moisture effect (k_{TMc}), moisture (k_{Mc}), and second order moisture effect (k_{Mc^2}), respectively.

$$\varepsilon = \varepsilon_0 + k_T T + k_{T^2} T^2 + k_{TMc} TMc + k_{Mc} Mc + k_{Mc^2} Mc^2 \qquad (9)$$

Correlation coefficients are given in Table 3 for grape skin, seeds and their mixture. Reported values are different from zero at a significance level of P<0.05.

Table 3 *Quadratic model coefficients (Eq. 9)*

	Milled mixture		Milled skin		Milled seeds	
	ε'	ε''	ε'	ε''	ε'	ε''
ε_0	231.80	7.79	20.83	11.21	80.78	24.61
k_T	-0.108	-	0.024	-0.033	0.699	0.171
k_{Mc}	-9.596	-0.361	-0.452	-0.383	-4.495	-1.419
k_{T^2}	-	-	-	-	-	-
k_{TMc}	-	-	-	-	-	-
k_{Mc^2}	0.107	0.006	0.009	0.005	0.065	0.021

Both the temperature and moisture content quadratic effects are negligible. The crossed effect of temperature and moisture is not clear either, as already reported in literature for a microwave drying process (*Tualsidas et al. 1995*). These results are in agreement with previous discussion.

3.4. Calculation of dielectric properties of the milled mixture from milled skin and seeds

To test the established measurement procedure, the following calculations were performed, and results were compared:

1) The dielectric properties of milled grape marc (seeds and skin mixture) were measured at a porosity of 0.4-0.6. These values were extrapolated with CRIM equation to zero porosity, and are shown in Figure 3, with the reference “Experimental”.

2) The same procedure was used to estimate zero porosity values for separated grape skin and seed samples. From these values, and the known ratio of skin (58 wt%) and seeds (42 wt%) in

the grape marc, the CRIM equation was used at zero porosity to estimate the dielectric properties of the mixture by combining the contributions of seeds and skin (with the reference "Estimated" in Figure 3).

The dielectric property values of grape marc at zero porosity obtained by procedures 1) and 2) are compared in Figure 3 as a function of moisture content for three different temperatures. Dielectric constant and loss both increase with increasing moisture content, as expected. In the case of lower moisture content the estimation from CRIM for seeds and skin was too great, while at higher moisture the same calculation underestimated the "experimental" values. The percentage standard deviation between 'experimental' and 'estimated' values was 17%. This level of precision was similar to that previously obtained when correlating milled gra marc as a function of porosity with the CRIM equation (Table 1), which was considered acceptable for this natural by-product material as a general purpose model. The agreement between both procedures may assure that the obtained values are close to the dielectric properties of the grape marc itself despite of the complex difficulties in the measurement method.

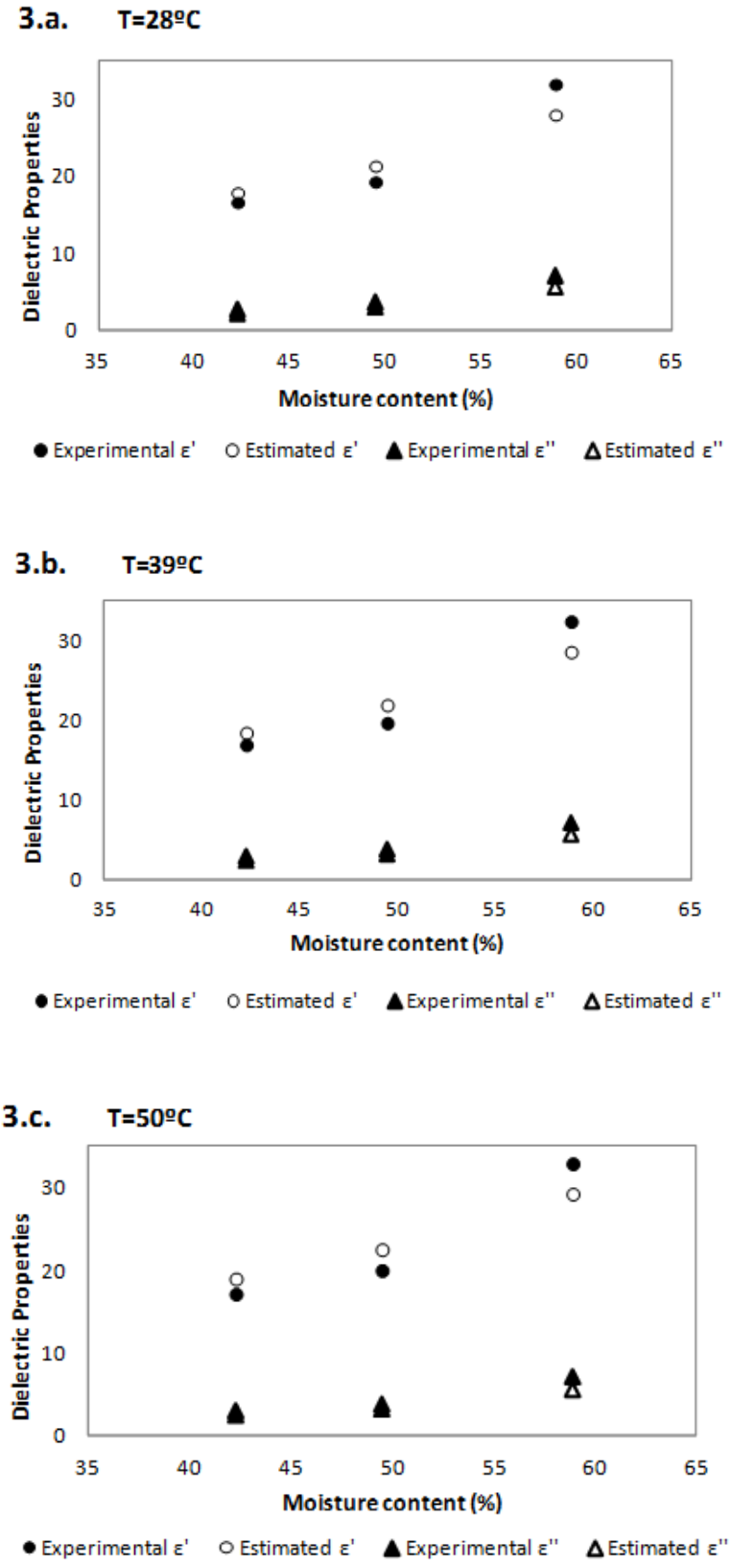

Figure 3 *Comparison of dielectric properties of milled grape marc at zero porosity calculated from the extrapolation of grape marc experimental values ('Experimental'), and from the estimation with the CRIM equation from seeds and skin constituents ('Estimated'). a) T=28 ºC b) T=39 ºC c) T=50 ºC*

4. CONCLUSIONS

Complex dielectric properties of grape marc and its separated macroscopic constituents (grape skin and seeds) were measured as a function of temperature (28-50 ºC) and moisture content (8.6 – 65.9 wt%). In previous studies, fresh, seedless grapes were measured using the coaxial line method, which requires perfect physical contact between the probe and the material. Here, the cavity perturbation method was used for the semi solid material as an "air-particle" mixture, after a milling step for sample preparation.

In order to obtain the dielectric properties of the material itself (air-free), different porosities were studied and mixing equation models were fitted to the results in order to obtain the dielectric properties at zero porosity. The complex refractive index mixing equation model (CRIM) was chosen because it provided the best fit between 0.4-0.6 porosity. The study of the influence of temperature and moisture was performed in this range, and experimental values were normalized to a porosity of 0.5 with the CRIM model.

This same mixing equation was used to calculate dielectric properties of grape marc from the experimental values of their individual constituents, seeds and skin, confirming the reliability of the measurement method (percentage standard deviation ≈ 17%).

In addition, correlation equations were obtained in order to calculate the dielectric properties of grape marc, seeds and skin as a function of moisture and temperature. As expected, moisture content had a significantly increasing effect on the dielectric properties for all samples. However, temperature was not shown to have a clear influence on either the dielectric constant or on the dielectric loss. This behaviour was attributed to the presence of salt in the grape marc.

The so obtained results will provide useful information in the design of a microwave cavity in order to maximize the treatment efficiency of grape marc in a microwave industrial extraction process. The use of reliable experimental values is essential when performing process scaling up from laboratory scale in order to consider the interaction between the complex material and the electromagnetic field.

ACKNOWLEDGEMENTS

The authors thank the Spanish Ministry of Economy and Competitiveness for projects CTQ2010-15475 and FracBioFuel ENE2012-33613 for funding. Katalin Solyom thanks the University of Valladolid for the financial support of the ”Estancias breves FPI UVa” Scholarship, and the Institute of Process Engineering in Life Sciences Section I: Food Process Engineering, Karlsruhe Institute of Technology for their technical support.

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Chapter 4.

Effect of absorbed microwave energy in phenolic compound extraction kinetics from grape marc

EFFECT OF ABSORBED MICROWAVE ENERGY IN PHENOLIC COMPOUND EXTRACTION KINETICS FROM GRAPE MARC

Abstract

Grape marc, a by-product of wine production, is a good candidate for the extraction of bioactive compounds, which are of great interest for the food, pharmaceutical and cosmetic industries. The long processing times and excessive solvent amounts required in conventional processes leave room for new extraction techniques like microwave assisted extraction. In this study, the extraction kinetics of phenolic compounds were described by a first order model at 80-150-300 W power levels, and three different absorbed energy densities. Complete extraction of total phenols was achieved at 200 J/ml of absorbed energy density, while anthocyanin was quickly extracted at any energy level. The extraction kinetics were 4 and 4.5 times faster for total phenol- and anthocyanin content respectively, compared to the conventional process. The cost of energy required for microwave treatment resulted in in 0.03 €/kg wet grape marc.

1. INTRODUCTION

Microwave assisted extraction (MAE) techniques have been investigated for more than 25 years, beginning with extraction protocols prior to chromatographic analysis *(Ganzler et al., 1986)*. The microwave technique was more effective than conventional procedures due to its rapidity and energy efficiency. The success behind microwaves is based on the heating theory of the oscillating electromagnetic field in a frequency range between 0.3 and 300 GHz. Heat is generated by the friction of molecules inside the material due to the reorientation of dipoles in the changing electric field. In industrial and domestic applications a frequency of 2.45 GHz is commonly used, providing 0.94 J/mol energy with a wavelength of 12.2 cm *(Letellier and Budzinski 1999)*. Water, an abundant component in natural raw materials, changes its orientation 4.9 109 times per second, producing rapid heating. This effect is enhanced by the contribution of ionic components and other substances with low specific heat *(Schubert and Regier 2005)*. The most pronounced advantages of microwaves in extraction processes are related to the rapid, volumetric and selective heating, thus resulting in high extraction efficiency from ecological and economic points of view. There is no direct contact between the energy source and the target material, major safety issues are not implied in the process, and moderate costs can be achieved by appropriate and efficient equipment design *(Letellier and Budzinski 1999, Chan et al 2011)*.

Numerous extraction systems were suggested for the enhanced extraction of active compounds with microwave energy, including open or closed equipment designs either in vacuum, atmospheric or inert environment, or under pressure *(Letellier and Budzinski 1999, Xiao et al., 2009, Chan et al., 2011, Huma et al., 2011b, Michel et al., 2011)*.

Short extraction times allow for the use of high temperatures, even for active compounds with low stability, in order to achieve high extraction yields. Satisfactory results have been reported for antioxidants from different natural matrices, without significant degradation *(Letellier and Budzinski 1999)*. Temperatures of up to 135ºC were reached in MAE in order to extract antioxidants from mandarin peels (Ahmad et al., 2012), while avoiding the Maillard reaction for 3 minutes. Liazid and co-workers (*2011*) found MAE suitable at 100 ºC for 5 minutes in order to extract anthocyanins from grape skin prior to chromatographic determination. Above this temperature anthocyanin degradation was observed. The absence of a solvent in the microwave processes also allowed for the enhanced anthocyanin extraction from sweet cherry, where 115ºC was reached in 45 seconds (*Grigoras et al., 2012*). A better antioxidant yield was

also obtained from solvent free extracts of buckthorn berries after 50 seconds of microwave treatment, always below 185ºC (*Michel et al., 2011*). In solvent free processes, the microwave energy is absorbed by the material with high moisture content, causing the possible disruption of vacuoles and cell walls. Thus, the antioxidants from intracellular spaces become more accessible, as was demonstrated in the case of onion by-products after a solvent free microwave hydro diffusion process (*Huma et al., 2011a, b*.)

The extraction of phenolics using MAE processes with solvents has so far been primarily performed on dried and milled raw materials to study the effect of solvent concentration, solvent-solid ratio, temperature, power and time, and to establish optimal extraction conditions (*Hayat et al., 2009, Ballard et al., 2010, Yang & Zhai , 2010, Song et al, 2011, Li et al., 2012a, Wu et al., 2012*). When small samples sizes are used, as in analytical procedures (*Liazid et al. 2011, Wataniyakul et al., 2012*), the cost of sample drying and milling is not a matter of interest. However, when enhanced industrial extraction is the target, drying and milling of raw materials could significantly increase the operating costs.

When microwave treatments are applied on raw materials with high moisture content, the proper water content can be used for extraction. The so called solvent-free microwave extraction (SFME) technique was first applied to the essential oil extraction from plants (*Lucchesi et al., 2007, Navarrete et al., 2011*). Based on these successful applications, the opportunity to gain antioxidants from fresh fruits (*Michel et al., 2011*) or from by-products of the food industry (*Huma 2011 a, b*) were also recently studied. Microwave heating was also used to enhance microbial decontamination and stability of apple purée, while maintaining the positive features of the product (*Picouet et al., 2009*).

By-products from wine production were found to be good candidates for the extraction of bioactive compounds, such as flavonoids, anthocyanins and stilbene derivatives, and are of great interest for the food, pharmaceutical and cosmetic industries (*Nassiri-Asl and Hosseinzadeh 2009*). However, the drawbacks of conventional extraction procedures (maceration and stirred extraction), such as long processing times and the requirement of excessive solvent amounts in mild conditions, leave room for new extraction techniques seeking a more efficient recovery process *(Spigno & De Faveri 2007, Spigno et al. 2007, Amendola et al. 2010, Lafka et al. 2007*).

Compared to other extraction techniques, MAE was proposed as one of the most effective procedures for the recovery of antioxidants from grape residues (*Casazza et al., 2010*),

although claims of higher yields using super-heated liquid extraction has been cited elsewhere (*Peralbo-Molina et al., 2012*). The extraction of fungicide residues from grapes for analytical purposes permitted more sensitive and faster performance with MAE than with solid-liquid extraction, or with matrix solid-phase dispersion (*Lagunas-Allué et al., 2012*).

The kinetics of the MAE process were described by a first order model in the case of the phenolic compounds of tea (*Spigno and De Faveri 2009*) and cocoa leaves (*Chan et al., 2013*), taking into account the absorbed microwave energy. On the other hand, anthocyanin extraction from grape peel was found to follow a Gauss function (*Li et al., 2012b*).

The aim of this study was to evaluate the extraction kinetics of phenolic compounds, and especially of anthocyanins from grape residue produced by the wine industry using two different microwave processes: SFMP (solvent-free microwave pre-treatment, prior to extraction) and MAE (microwave assisted extraction). In the case of MAE, different levels of absorbed microwave energy and power were studied in order to evaluate their influence on the extraction rate constant and the final extraction yield of total phenol- and monomeric anthocyanin content. Results were compared with a patented conventional extraction process (*Moro-Gonzalez 2010*). For industrial considerations 1) the raw material remained untreated prior to the modified extraction processes, 2) the energy absorbed was evaluated in the microwave processing, and 3) the cost of energy in the intensified process was calculated.

2. MATERIALS AND METHODS

2.1. Raw material

A mixture of red grape seed and skin (grape marc) from the region of Toro (Spain) was used in the experiments. In the wine production, the grape marc was separated from the liquid phase after the red wine. 10 kg of grape marc was distributed in 100 g packages and stored at -18 °C until the time of the experiment. Defrosting was performed at 4 °C overnight, the day before the extractions.

2.2. Extraction techniques

The effect of microwave irradiation on the extraction kinetics was studied using two methods. On one hand microwave assisted extraction was applied, in which the mixture of raw material and solvent was exposed to the microwaves. On the other hand, solvent-free microwave pre-treatment was used to achieve cell wall rupture in the raw material in the absence of a solvent,

and further continue with a conventional stirred extraction. The results of both techniques were compared to a conventional patented method (*Moro-Gonzalez 2010*), following referred to from now on as the "control"

2.2.1. Microwave assisted extraction (MAE)

MAE experiments were carried out in a piece of laboratory microwave equipment (CEM® Discover) with a maximum output power of 300W. 30 g of wet grape marc was placed in a 100 ml round-bottomed flask, open to the atmosphere. The material was gently stirred with a mechanical stirrer at 50 rpm to facilitate irradiation homogeneity in the material. Although the device is equipped with an infrared thermometer, the temperature inside the flask was measured with a fibre optic temperature sensor during the treatment (FoTemp 4, OPTOcon GmbH). Microwave irradiation was performed at constant power (80, 150 and 300 W). At every power level, three irradiation times were used (Table 1).

These irradiation times were chosen in order to supply similar absorbed energies at the three power levels. Before microwave irradiation, 60 ml of 1:1 volumetric mixture of ethanol (96%) and acidified water (pH=1) was added to the grape marc as the extraction solvent. After stirring for 1 minute without microwave irradiation, the first sample of 1.5 ml was taken as an initial value for the kinetic analysis, and the initial temperature was recorded.

After microwave irradiation, the final temperature was recorded again, and a second sample was taken for further analyses. After irradiation, stirring continued for 20 minutes of the total extraction time, with periodic sampling from the extraction vessel. During the whole extraction, a total of five samples were gained and filtered through 0.2 µm PET syringe filters for further analyses of the total phenol content and antioxidants. Every treatment was performed in duplicate from the same batch of grape marc.

Table 1 Experimental conditions in MAE: power levels and irradiation times

Power (W)	**80**	**150**	**300**
Irradiation time (s)	80	40	20
	160	80	40
	240	120	60

2.2.2. Solvent-free microwave pre-treatment (SFMP)

The experimental setup was prepared as in Section 2.2., with the difference that the solvent was added after instead of before the microwave treatment. Right after irradiation, samples were cooled down in a cold water bath, and placed into a bath to continue the extraction at 40 ºC, as described in Section 2.2.3.

As a preliminary test, 150W of power was applied for 55 and 80 s on grape marc with an 85% moisture content. The effect of moisture content was also studied by pressing juice out of the grape marc before the treatment, in order to reduce the moisture content from 85% to 73%. Samples from the solvent free pre-treated extraction process were compared to "control" samples, without microwave pre-treatment, as described in section 2.2.3. Experimental error was obtained from duplicate experiments.

2.2.3. Conventional solid- liquid extraction

The same glass vessel with mechanical stirring was used, as described in the MAE experiments (but without microwave treatment) and was placed directly into a water bath at 40°C. The same sample and solvent amounts were also used, as in the MAE experiments. 1.5 ml samples were taken for kinetic analysis after 10-20-45-90-180 min and filtered through 0.2 µm PET syringe filters for further analyses of the total phenol content and antioxidants. Extractions were performed in duplicate.

2.3. Absorbed energy calculation

In microwave processes, the product of the applied power multiplied by the irradiation time determines the energy emitted by the device. However, this amount of energy is not completely absorbed by the material. The fraction of energy absorbed by the sample will depend on the dielectric and geometric characteristics of the material, as well as on the type and geometry of the microwave oven. To take advantage of the results presented here, so that they can be transferred to other devices and setups at laboratory or industrial scale, it is necessary to estimate the fraction of energy absorbed by the grape marc and the solvent mixture. This magnitude has been calculated in terms of density of theabsorbed energy ($E_{absorbed}$ *[kJ/ml]*) from an energy balance with three contributors (Eq. 1) (*Sólyom et al., 2011*):

$$E_{absorbed} = Q_{sensible} + Q_{latent} + Q_{loss} \quad (1)$$

At the beginning of the irradiation period, most of the energy is spent on heating the sample, as sensible heat ($Q_{sensible}$). This term can be calculated from the mass of grape marc plus the solvent mixture (m_{sample} *[g]*), the temperature increment in the microwave treatment (ΔT *[ºC]*), and the specific heat capacity of the mixture of grape marc and solvent (*Cp = 3.555 [kJ/(kg*K)]*) (Eq. 2). The heat capacity of the grape marc at certain moisture content was estimated as described by Tulasidas (*1994*). The sensible heat term:

$$Q_{sensible} = m_{sample}\, Cp\, \Delta T \qquad (2)$$

When the temperature reaches the boiling point of the mixture, the energy is used up on strong evaporation as latent heat (Q_{latent}), which can be determined as the product of the weight of the evaporated solvent and the latent heat of its vaporization. The third element in the energy balance (Q_{loss}) takes into account the heat loss from the vessel surface into the environment during the treatment, which can be determined through the heat transfer coefficient of the system, obtained by the decrease of temperature over time of a hot sample placed into the microwave cavity without microwave irradiation (*Sólyom et al., 2011*).

In the present case, temperatures below the boiling point were achieved and no solvent losses were observed, therefore the evaluation of the latent heat was not performed. Due to the short irradiation time, and the lack of free volume inside the cavity, the heat loss from the vessel surface was negligible. This simplification was checked experimentally. Thus, Eq. (1) can be simplified as follows:

$$E_{absorbed} \approx Q_{sensible} = m_{sample}\, Cp\, \Delta T \qquad (3)$$

2.4. Moisture content analysis

The moisture content of the grape marc samples was determined before the microwave treatments in the case of every new opened 100 g pack. Samples were dried at 105°C for 24 hours. From the wet and dry masses the moisture content was calculated. Analyses were performed in duplicate for every sample.

2.5. Total phenol content analysis

Total phenol content (TPC) was quantified, using the Folin-Ciocalteu method, as Gallic acid equivalents per gram of dry material [*mg GA/g DM*] (*Singleton et al. 1999*). A volume of 40 µl of filtered sample was diluted with distilled water (3 ml) and mixed with 200 µl of Folin-Ciocalteu reagent. After 10 minutes, saturated Na_2CO_3 was added to the solutions, and the

samples were incubated at 40ºC for 30 min. Absorbance was measured at 765 nm (UV 2550 Shimadzu UV/VIS spectrometer) and known concentrations of diluted Gallic acid were used for calibration.

2.6. Anthocyanin content analysis

Total monomeric anthocyanin pigment content was determined by the pH differential method (*AOAC 2005.02*). Results are expressed on the basis of cyanidin-3-glucoside [*mg cyanidin-3-glucoside/ g dry matter*].

2.7. Extraction kinetics model and data analysis

In several occasions the extraction was found to follow kinetics of first order (*Spigno and De Faveri., 2009, Chan et al., 2013*), which was also suggested in the present study.

$$Y(t) = Y_0 + Y_f(1 - \exp(-\beta t)) \quad (4)$$

In Eq. (4) *Y(t)* refers to the extraction yield of TPC (Y_{TPC}*; [mg GA/g DM]*) or anthocyanins (Y_{AC}*; [mg cyanidin-3-glucoside/ g DM]*) at a certain time (*t; [min]*). The sum of the initial yield (Y_0) and the pre-exponential constant (Y_f) results in the maximal extraction yield (Y_{max}) at infinite time. The extraction rate constant (β [min^{-1}]) can also be expressed separately for TPC (β_{TPC}) or Anthocyanin content (β_{AC}).

The parameters of the extraction kinetics were calculated using Statgraphics Centurion XVI Software with the Gauss-Newton non-linear regression method. Standard deviation and P= 0.05 confidence limits were also determined.

3. RESULTS AND DISCUSSION

3.1. Absorbed energy evaluation and efficiency

Irradiation times were chosen with the aim of supplying the same absorbed energy at different power levels. However, when the absorbed energies were evaluated according to the sensible heat transferred to the sample, small discrepancies were observed in some cases (Table 2). The three levels of energy absorbed by the samples were 18.4±0.7, 12.3±0.5 and 6.1±0.2 kJ, with absorption energy efficiencies related to emitted energies of 90%±2%, 90%±7% and 98%±2%, respectively. The lowest energy value (6.1±0.2 kJ) was completely absorbed by the system (98%±2%). At higher energy levels (>12 kJ), the energy absorption was lower (90%), but still much more efficient than in experimental setups with domestic microwave ovens (Sólyom et

al., 2011). The absorbed energy density ($E_{density}$ *[kJ/ml]*) can be calculated from the determined absorbed energy and the volumes of sample and solvent.

Table 2 Absorbed energy evaluation in MAE experiments

Power	*Time*	T_{final}	ΔT	$E_{emitted}$	$E_{absorbed}$	$E_{density}$	*Efficiency*
W	*sec*	*°C*	*°C*	*kJ*	*kJ*	*kJ/ml*	*%*
80	240	72	57	19.2	17.2	0.201	90%
80	160	57	41.5	12.8	12.5	0.147	98%
80	80	35	21	6.4	6.3	0.074	99%
150	120	70	55	18.0	16.6	0.194	92%
150	80	49	35.5	12.0	10.7	0.125	89%
150	40	34	19.8	6.0	6.0	0.070	100%
300	60	67	52.5	18.0	15.9	0.186	88%
300	40	48	33.5	12.0	10.1	0.118	84%
300	20	34	19	6.0	5.7	0.067	96%

Most literature on antioxidant extraction with the assistance of microwave energy deals with various experimental conditions, and also makes use of different microwave oven types, sizes and geometries. On a few occasions, attention has been given to the importance of power density (W/g or ml sample) or energy density (J/g or ml sample) (*Huma et al., 2011 b, Li et al., 2012 b, Hiranvarachat et al., 2013, Chan et al., 2013*). Nevertheless, the method used to quantify the absorbed energy has barely been described. Energy characterization was performed in the case of antioxidant extraction from dry cocoa leaves (*Chan et al., 2013*) and of β-carotene extraction from carrots (*Hiranvarachat et al., 2013*), but no determination has been found for the extraction from grape residues so far.

3.2. Preliminary study on SFMP

Pre-treatments performed at 150 W without solvent were compared in terms of irradiation time and sample moisture content. No relevant differences were observed in any of the cases, neither in total phenol content nor in extraction kinetics, between the control and SFMP experiments (Figures 1a and 1b). In the analysis of irradiation time (Figure 1a) both experiments were compared to a control extraction, made on the same day from the same sample charge ("Control 1", "Control 2"). The tendency observed for total phenol content was similar to that of anthocyanin content (data not shown). Likewise, no difference was observed when different moisture contents were compared with the corresponding control extractions after microwave irradiation (Figure 1b). A higher yield of total phenol content was obtained in the case of grape

marc with 85% moisture. This difference is explained by the loss of active compounds after pressing, which reduces the moisture in the raw material. As in the former case, the anthocyanin content showed similar evolution to the total phenol content. These unsuccessful results led to the discarding of SFMP as a possible intensification process, although for other raw materials positive results were obtained elsewhere (*Michel et al., 2011, Huma et al., 2011 a, b*).

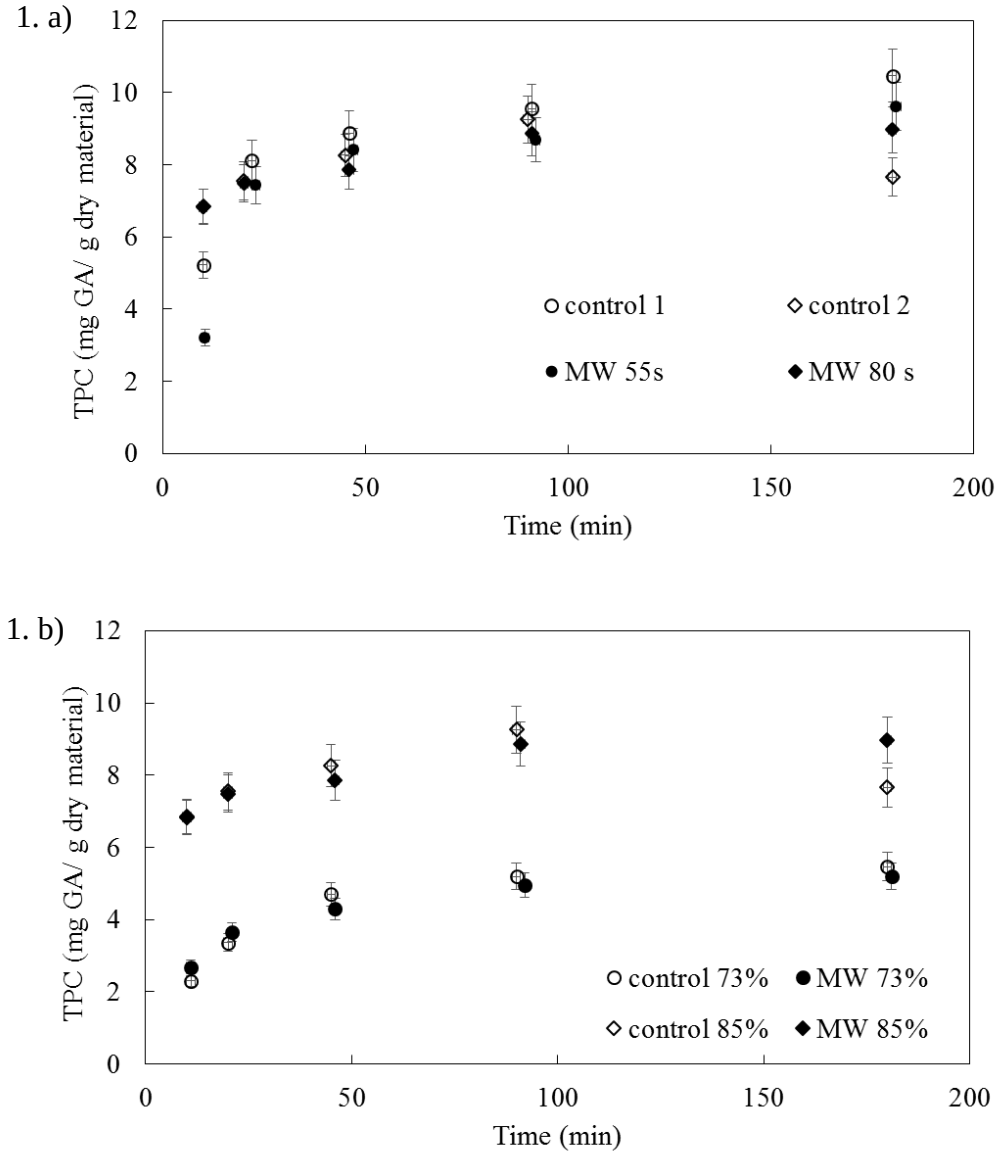

Figure 1. Total phenol content evolution of extract during conventional solid liquid extraction (control) and after SFMP treatments at 150 W: a) with irradiation times of 55 and 80 s, and b) with samples with different moisture contents (73 %, 85%).

3.3. First order kinetic model fit of experimental data

3.3.1. MAE experiments

Parameters of the first order kinetics extraction for MAE and control experiments are listed in Table 3, including standard deviations and R2 coefficients. The average coefficients of determination (R^2) were 95.8 and 94.2% for TPC and anthocyanin measurements respectively, showing good correlation with the experimental data.

From Y_0 and Y_f values, the maximum yield of TPC ($Y_{max\ TPC} = Y_0+Y_f$) was calculated and represented at different power and absorbed energy levels in Figure 2. Increasing treatment energy at every power level caused increased extraction yields, however only the highest energy level (≈ 200 J/ml) rendered the same yield as the control samples. A significant influence of power cannot be perceived from experimental results, although the increment in yield with increasing treatment energy (the slope) is more pronounced at 80 W than at 300 W. This effect is probably due to the longer irradiation time required at lower power.

Table 3. First order kinetic parameters for MAE and control extraction experiments.

Power	E density	Yo_{TPC}	σ (Yo)	Yf_{TPC}	σ (Yf)	β_{TPC}	σ (β)	R^2	Yo_{AC}	σ (Yo)	Yf_{AC}	σ (Yf)	β_{AC}	σ (β)	R^2
W	kJ/ml	mg GA/g DM		mg GA/g DM		min^{-1}		%	mg CG/g DM		mg CG/g DM		min^{-1}		
80	0.201	1.75	0.38	7.75	0.37	0.181	0.024	96.3	9.73E-04	2.0E-04	1.85E-03	1.9E-04	0.418	0.076	91.4
80	0.147	2.89	0.32	5.32	0.31	0.186	0.032	97.8	1.57E-03	2.7E-04	1.66E-03	2.5E-04	0.216	0.088	87.5
80	0.074	2.54	0.29	3.10	0.28	0.233	0.053	88.7	1.46E-03	8.5E-05	1.01E-03	8.6E-05	0.184	0.046	89.1
150	0.194	1.84	0.59	7.04	0.56	0.220	0.048	91.2	1.06E-03	1.9E-04	2.05E-03	1.8E-04	0.357	0.061	92.6
150	0.125	1.28	0.41	5.84	0.38	0.341	0.043	98.1	7.63E-04	1.9E-04	2.16E-03	1.8E-04	0.402	0.059	97.3
150	0.070	2.89	0.26	3.72	0.25	0.256	0.044	97.5	1.63E-03	1.6E-04	1.43E-03	1.5E-04	0.276	0.069	94.8
300	0.186	0.68	0.44	6.50	0.41	0.310	0.042	98.3	3.98E-04	1.3E-04	1.98E-03	1.2E-04	0.560	0.051	99.2
300	0.118	1.42	0.61	5.26	0.57	0.370	0.073	96.2	9.23E-04	1.9E-04	1.71E-03	1.8E-04	0.459	0.075	97.3
300	0.067	2.42	0.26	3.57	0.24	0.272	0.043	97.9	1.16E-03	6.7E-05	1.21E-03	6.2E-05	0.337	0.035	99.1
Control	-	1.97	0.77	7.96	0.36	0.057	0.010	94.5	9.23E-04	1.2E-03	2.27E-03	1.0E-04	0.090	0.018	89.6
Control	-	1.97	0.77	6.72	0.44	0.113	0.031	75.7	9.23E-04	1.2E-03	2.01E-03	5.0E-05	0.164	0.023	88.7

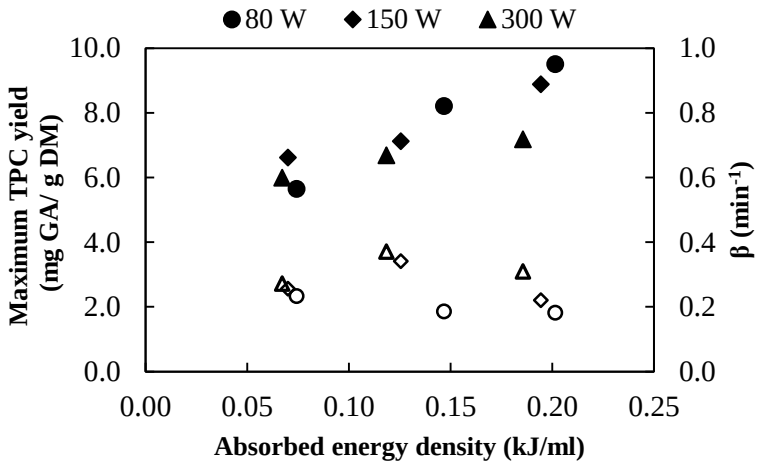

Figure 2. First order parameters (Y max: full bullets; β: empty bullets) of TPC at different power and energy levels in MAE experiments

The other key parameter in extraction kinetics is the rate constant, β. The higher the value of β, the faster the extraction kinetics. In the case of TPC values, the rate constant seems to show a maximum value versus increasing energy. The analysis of total phenols provides a global view of possible valuable compounds. These phenolic compounds are located at different cell structures within the material, with different degrees of accessibility. A possible explanation of the observed maximum in the kinetics could be based on these differences in accessibility: with higher absorbed energy, the phenolics that are more difficult to extract become available, and the maximum yield is increased, but their kinetics of extraction are lower, reducing the global kinetics of the process. The use of higher power levels provided a faster extraction, as the temperature increment in the sample is faster at higher irradiation intensities.

In the case of anthocyanin content (Figure 3) maximum extraction yields did not change significantly with increasing irradiation energy or power level. Compared to control samples, final yields are similar or even lower after MAE experiments. As regards the extraction rate constant of anthocyanins, higher values were obtained at higher energy levels. Similarly to the TPC, the higher was the applied power, the higher was the rate constant in the extraction.

The energy amounts studied here are in accordance with those published for cocoa leaves (*Chan et al., 2013*), as the extraction was insufficient below100 J/ml and equilibrium was found between 100-300 J/ml. Higher energies were not studied in order to avoid high temperatures, excessive evaporation, and insufficient precision in the determination of absorbed energy.

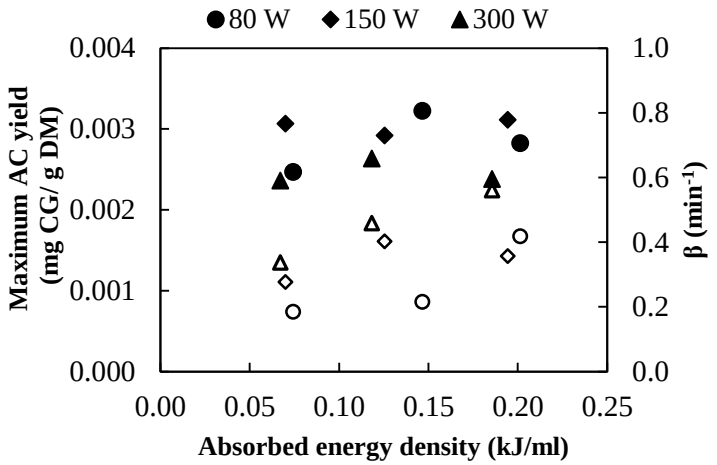

Figure 3. First order parameters (Y max: full bullets; β: empty bullets) of anthocyanins at different power and energy levels in MAE experiments

The MAE experiments presented here were not performed in the usual manner, since here the shorter microwave irradiation stage was followed by an insulated stirred extraction stage at the temperature achieved during the irradiation. Using the first order kinetic model, the extraction yield obtained right after the irradiation pre-treatment, before stirred non-irradiated extraction, can be determined. Figure 4 shows the thus calculated relative yields for TPC and anthocyanin content (AC) at different power levels, corresponding at the highest energy level (200 J/ml). In all cases, the recovered percentage of Y_{max} was greater when lower power levels were used.

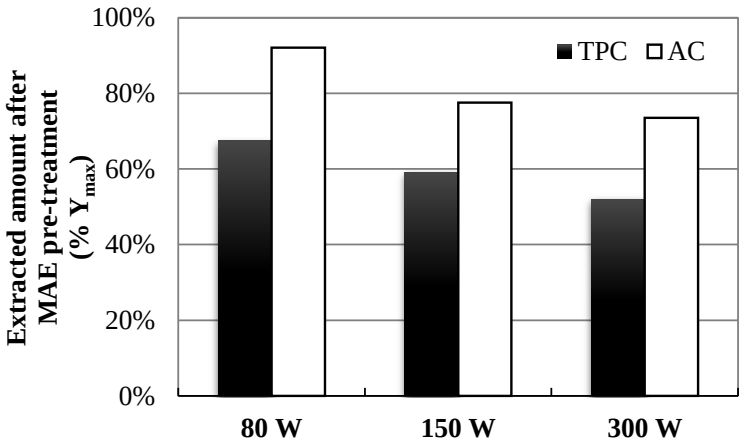

Figure 4. Relative extraction yield right after microwave irradiation in MAE experiments (Edensity≈200 J/ml)

The MW irradiation at a higher power level (300 W) causes the rapid heating of localized points in the experimental system, and the so-called hot spots are formed (*Chan et al., 2011*). When the irradiation stops, temperature inhomogeneity among the different points of the load is more likely to appear at higher power levels. During the following minutes of extraction, while the

temperature becomes homogenous and increases in the bulk, a further increment in yield can be observed. The extraction of anthocyanins were faster, and 74-92% of the final yield was obtained right after the microwave irradiation.

3.3.2. Conventional solid-liquid extraction (control experiment)

As in the "Control" experiment, extraction of grape marc was also performed without the presence of microwave irradiation. Mild extraction conditions at 40 °C resulted in a longer extraction process, which was observed for 3 hours to assure complete extraction of the studied compounds. The first order model for TPC (Figure 5) resulted in an average correlation of R^2= 85.1%, similar to that of the anthocyanin content (R^2=89.2%). To perform the correlation of these experiments, the values of $Y_{0,TPC}$ and $Y_{0,AC}$ were fixed at the average values taken from MAE experiments. Such an approximation was necessary because the first experimental points in the control extraction were gathered after 10 minutes. As the raw material was the same in every experiment, the values measured in MAE after 1 minute of extraction, before MW irradiation, were very close to the estimated Y_0 parameter. This justifies the use of the average initial yields for the control experiments since, in the first minute, products and conditions were indistinguishable between MAE and control experiments.

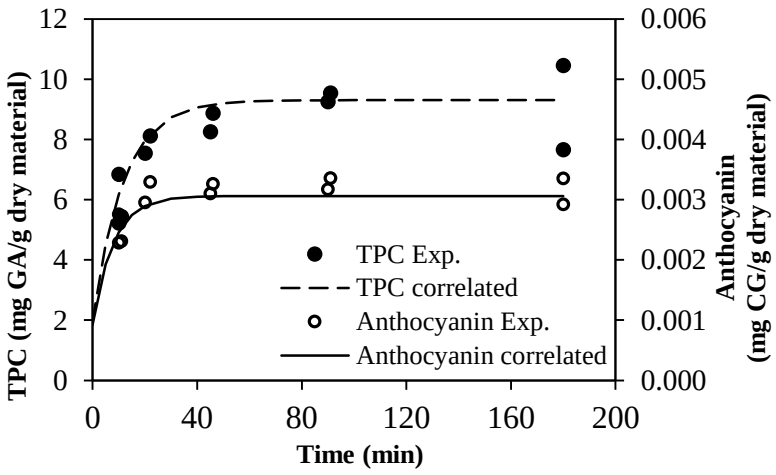

Figure 5. First order model fit of experimental data of TPC and AC for Control extraction

The TPC yield highly surpassed that of the anthocyanins, since the latter represented only a small fraction of all the phenolic in the grape. There was no change in the final yields after 80 and 50 minutes for total phenol-, and anthocyanin content, respectively, due to differences in the location of the compounds. Most of the anthocyanins could be found joined to the grape skin in vacuoles or in bound form with the cell membrane (*Pinelo et al., 2006*), thus being accessible for extraction. In grape marc, besides partially disintegrated skin, whole grape seeds

are present. The complex structure of the grape seed provides an additional rate-limiting step to mass transfer in extraction, which could explain the slower total phenol extraction kinetics compared to that of anthocyanin content.

3.4. Comparison of MAE to conventional solid-liquid extraction

The main objective of microwave treatment in an extraction process is the reduction of the long extraction times of conventional processes under mild experimental conditions. For this reason, a comparison was made between the conventional method and the most effective microwave treatment ($E_{density}$ ≈ 200 J/ml), at different power levels. Figure 6 shows the results for this comparison in terms of the extraction time required to achieve 98% of the maximum yield ($t_{98\%}$). For both the TPC and the anthocyanin content, the required time is greatly reduced in comparison with the control sample, showing the effective intensification of the process, while the maximum yield does not change significantly regards the anthocyanin content.

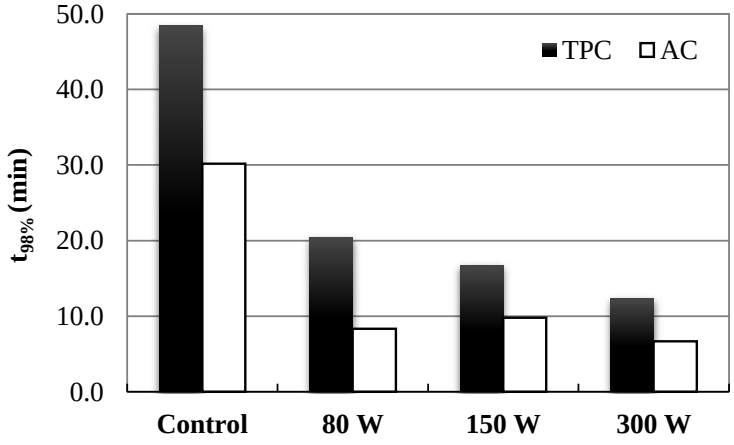

Figure 6. Required time to achieve 98% of Y_{max} in control and MAE experiments ($E_{density}$≈200 J/ml) at different power levels

In other studies, similar results have been observed, as the MAE process did not increase the final yield, but definitely lowered the process time compared to the conventional method (*Picouet et al., 2009, Peralbo Molina et al., 2012, Lagunas-Allué et al., 2012, Hiranvarachat et al., 2013*). In contrast, others found that MAE achieved higher extraction yields (*Proestos and Komaitis, 2008, Hayat et al., 2009, Huma et al., 2011 a,b, Ballard et al., 2010*). These differences may be due to the diversity of the plant tissues in antioxidant extractions, and also the conditions of the comparative conventional methods.

There is a decreasing trend in the $t_{98\%}$ values with increasing microwave power. The longer irradiation times necessary at lower power to achieve the same absorbed energy may explain this phenomenon.

3.5. Energy cost

The MAE pre-treatment process evaluated here is able to reduce the extraction time by 4 and 4.5 without significant losses of extracted compounds for TPC and AC respectively. From an industrial point of view, there is an advantage in reduced reactor sizes and reduced retention times, resulting in reduced ethanol stocks, which is a critical economic issue. The use of microwaves essentially implies electric energy. Assuming from Figures 2 and 3 an estimated value of 16 kJ for 30 g wet grape marc, with 70% efficiency in the conversion of electric power into microwave power, and a price of 0.15 €/kWh, an approximate operating cost of 0.03 €/kg wet grape marc can be obtained, excluding the cost of equipment.

4. CONCLUSIONS

The effect of microwave irradiation was studied in order to enhance the extraction kinetics of polyphenols from grape marc. A preliminary study of a solvent-free microwave pre treatment (SFMP) did not show any enhancement in the extraction, and thus hereinafter only microwave assisted extraction (MAE) was studied. After a short irradiation time (max 4 minutes) of the mixture of solvent and fresh grape marc, extraction was continued up to 20 minutes, and samples were taken for kinetic analyses. First order kinetics were found to be adequate to follow the extraction yield evolution in terms of total phenol and monomer anthocyanin content. Complete extraction of polyphenols was achieved at 200 J/ml absorbed energy density and the extraction time was reduced 4-fold when compared to a patented conventional method. Concerning the anthocyanin content, the extraction yield did not significantly change at any energy level, but the time required to achieve 98% of maximum yield was 4.5 times shorter than in conventional solid-liquid extraction. Energy cost evaluation reported a value of 0.03 €/kg wet grape marc, a moderate cost for such an intensified extraction process, where solvent stock requirements and extraction times are sharply reduced.

ACKNOWLEDGEMENTS

The authors thank the Spanish Ministry of Economy and Competitiveness for the CTQ2010-15475 Project and the ENE2012-33613 Project. Katalin Sólyom acknowledge the University of Valladolid for the financial support of the "Estancias breves FPI UVa" Scholarship.

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Chapter 5.

Enhanced polyphenol extraction of grape marc after ultrasound pre-treatment

ENHANCED POLYPHENOL EXTRACTION OF GRAPE MARC AFTER ULTRASOUND PRE-TREATMENT

Abstract

In this study ultrasound (US) was applied as a pretreatment, followed by conventional extraction of polyphenols, in order to enhance extraction kinetics using a lower energy input than other ultrasound assisted extraction processes, seeking for possible implementation on an industrial scale. The antioxidant extraction from grape marc, a by-product of wine production, is constantly gaining more interest in food, pharmaceutical and cosmetic industry. Ultrasound assisted extraction is known to enhance the slow mass transfer stage, inherent in the conventional extraction processes. The influence of absorbed energy and energy intensity were evaluated. Increasing temperature (40-55-70°C) caused strong improvement, while energy intensity (100-75-50%) showed little influence on the final extraction yield and on the initial extraction rate. Up to 70% of the final yield was obtained solely through US pretreatment. Finally, only the thermal effect of US energy was confirmed by comparison of thermal and US pretreatments, and no clear evidence of a specific ultrasound effect could be verified.

1. INTRODUCTION

Worldwide annual grape production is over 65 million tons, from which more than 25 million tons of wine is produced (*FAOSTAT, 2010*). During the wine-making process almost the same amount of semi-solid waste is generated, called grape marc, which contains the grapes' skin, seeds, and the wine lees after the fermentation. During the fermentation process of red wine a mild extraction of the grape skin and seed occurs, thus improving the color and polyphenol profile of the wine. The remaining residue is still rich in antioxidants, which is desirable to eliminate in order to avoid inhibition problems before using the grape pomace as fertilizers (*Negro et al. 2003, Pinelo et al. 2006*). The extracted bioactive compounds mentioned (flavonoids, anthocyanins and stilbene derivatives) are attractive products for the food, cosmetic or pharmaceutical industry, due to the high antioxidant, and antimicrobial, antiviral or anticarcinogenic effects (*Nassiri Asl and Hosseinzadeh 2009*) .

The widely used and studied conventional maceration or stirred extraction under mild conditions has the disadvantage of long processing times because of slow mass transfer, and requires huge solvent amounts (alcohols and water) which must be removed from the final product (*Spigno and De Faveri 2007, Spigno et al. 2007, Amendola et al. 2010, Karacabey and Mazza 2010, Lapornik et al. 2005*). Different sample pretreatments and novel extraction techniques were recently studied and compared in order to improve mass transfer and extraction kinetics of antioxidants, such as supercritical fluid extraction and microwave assisted extraction, Soxhlet-extraction or ultrasound assisted extraction (*Martino et al. 2006, Pascual-Marti et al. 2001, Casazza et al. 2010, Rodriguez-Rojo et al. 2012, Peralbo-Molina et al. 2012*). Positive effects of ultrasound assisted extraction (UAE) in food industry were reviewed by Vilkhu et al. (*2008*), who reports increased extraction yields and initial extraction rates.

The propagation of ultrasound (US) waves leads to cavitation phenomena. Compression and stretch of the molecular spacing cause the formation and violent collapse of cavitation bubbles in the liquid, thus increasing the mass transfer, causing surface peeling, erosion and particle breakdown. The cell disruption or enlargement of cell wall pores due to the cavitation leads to the washing out of the intracellular content. Cell plasmolysis and alteration of different cellular layers were observed on US treated grape berries, using light and transmission electric

microscopy (*Fava et al 2011*). In US systems the electrical energy is converted into mechanical vibrations at a constant frequency, which is then transferred to the medium in an ultrasonic bath or using an ultrasonic probe. The intensity of the US energy is proportional to the square of the amplitude where there is a required minimum in order to achieve cavitations in the medium, although excessively high intensities may cause the degradation of the desired compounds. The majority of the dissipated acoustic energy is converted into heat. The consequently increased temperature has a positive effect on the extraction but at the same time causes a less violent collapse of the cavitation bubbles as they are filled with solvent vapor at higher temperatures. For this reason, most of the UAE applications are performed in a thermostatic environment. The nature of the solvent and treatment cycles (continuous or pulsed US) are also important factors during the process (*Weiss et al. 2011, Santos et al.2009*). These parameters were studied and optimized for several raw materials in order to achieve the desired features in the extracted product (*Palma and Barroso 2002, Wang et al. 2008, Benito-Román et al. 2013, Londoño-Londoño et al. 2010, Morelli and Prado 2012*). Extraction kinetics were found to fit first order behavior (*Virot et al. 2010, Pingret et al. 2012, Karabegovic et al. 2011, Topallar and Gecgel 2000*.) although Naik´s model (*Carcel et al. 2010, Ahmad Quasem et al. 2012*) and second order kinetics (*Pan et al. 2011, Qu et al. 2010*) were also proposed for the modelling of extraction processes.

The efficiency of the UAE process is directly connected to the intensity of the acoustic energy supplied to the sample, which is usually designated as 'specific energy' (power/area of sonotrode). This specific energy corresponds to the dissipated acoustic power in the liquid, and must be distinguished from the power consumption of the US device. The latter can be measured by a high precision wattmeter, while the former is experimentally determined by calorimetry in an insulated vessel (*Weiss et al. 2011, Romdhane et al. 1995, Ratoarinoro et al. 1995, Kuijpers et al. 2002*). This specification of the energy input is indispensable in scaling up the UAE process (*Virot et al. 2010, Pingret et al. 2012*).

The aim of this study was to evaluate an effective low energy input ultrasound pretreatment, prior to the conventional industrial process for the extraction of polyphenols. No additional sample preparation steps (drying or mechanical particle size reduction) were applied to the raw material, because of their high operating cost in industrial scale. The extraction kinetics of polyphenols from fermented grape marc were measured after short US pretreatments, using different absorbed energy levels. The so obtained kinetic parameters were compared to those

from conventional extraction without pretreatment (Control). The operating variables in the US pretreatments were the final pretreatment temperature (40-55-70ºC) and the US amplitude (50-75-100%). Also the possible non-thermal US effects were studied by the comparison of analogous thermal and US pretreatments, following the same thermal history.

2. MATERIALS AND METHODS

2.1. Raw material

Fermented grape marc was received from the Matarromera winery (Spain) in November 2011. It was a mixture of pressed, partially ruptured grape skin and whole seed, which had contributed to the color development of the red wine during the fermentation process. For industrial reasons the samples had 1 g/kg grape marc $NaHSO_3$ content. Samples were stored in a freezer at -20ºC and defrosted at 5ºC overnight before the pretreatments and extraction processes.

The moisture content of the grape marc was determined in an oven at 105ºC after 24 hours drying. The original moisture content was around 60% for the milled skin and seed mixture.

2.2. Conventional extraction

A continuous stirred extraction was performed for 3 hours in a closed round-bottomed flask containing 30 g wet grape marc in 150 ml solvent of 50 v/v% ethanol and acidified water at pH=1 (Moro Gonzalez 2010). Temperature (40ºC) was maintained using a water bath. During the extraction 0.5 ml samples were taken at 5, 15, 30, 60, 120, 180 min to study the extraction kinetics of polyphenols. The samples that did not suffer any treatment prior to the extraction will subsequently be referred to as "control". Two parallel extraction setups were used for every treatment condition.

2.3. Ultrasound treatment and absorbed energy determination

Ultrasound was performed before the conventional extraction procedure as a pretreatment stage. A probe type ultrasound system was applied (UP400S; Hielscher GmbH, Germany) with constant frequency (24 kHz), a titanium sonotrode (D=22mm), maximum power of 400W and maximum amplitude of 100μm.

Pretreatments were carried out (30 g of wet grape marc and 150 ml solvent) in a double-walled glass vessel providing thermo insulation (air) which allowed the medium to heat up from room

temperature. The vessel was covered with aluminum foil to diminish the evaporation of the solvent, although the evaporated amount was measured by weight and replaced after the pretreatment. Additional magnetic stirring was carried out in order to avoid the settlement of the grape marc. After the pretreatment, the solution was rapidly cooled down to 40ºC and the extraction continued for 3 hours in the conventional stirred system, as it was formerly explained. The first sample for the kinetic study was taken just after the pretreatment, before the start of the conventional extraction.

The effect of ultrasound intensity was studied with different amplitudes (50-75-100%) and at different final temperatures (40-55-70ºC). The pretreatment time was set to reach the specified final temperature. Every pretreatment was carried out in parallel and the duplicate of the middle point (55ºC, 75%) was repeated 3 times.

The consumed power ($P_{consumed}$) of the system is distributed between the inefficiency in the generator and the transducer, and the dissipated acoustic power in the liquid. Only the latter represents the efficient (absorbed) energy in the treatment, since the others depend on the type of equipment (Weiss et al. 2011). The energy absorbed ($E_{absorbed}$ *[kJ]*) by the grape marc and the solvent mixture is finally transformed into heat. So, the absorbed energy may be calculated using an energy balance taking into account the temperature increment of the system ($Q_{sensible}$), the solvent evaporation (Q_{latent}) and the heat loss (Q_{loss}) through the vessel wall (Sólyom et al., 2011) (Eq. 1):

$$E_{absorbed} \approx Q_{sensible} + Q_{latent} + Q_{loss} \quad (1)$$

Sensible heat ($Q_{sensible}$ *[kJ]*) is calculated from the mass of the grape marc and the solvent mixture (m_{sample} *[g]*), the temperature difference (ΔT *[ºC]*) before and after the ultrasound treatment, and the specific heat capacity of the grape marc and solvent mixture ($Cp = 3.374$ *[kJ/(kg•K)]*). (Eq. 2) The heat capacity of the grape marc at certain moisture content was estimated as it was described by Tulasidas (*1994*).

$$Q_{sensible} = m_{sample}\, Cp\, \Delta T \quad (2)$$

Latent heat of vaporization (Q_{latent} *[kJ]*) is calculated from the amount of evaporated solvent (m_{vap}), measured by weight loss after microwave treatment, and the heat of vaporization of the solvent (ΔH_{vap}*= 540 [kJ/kg]*) (Eq. 3).

$$Q_{latent} = m_{vap}\,\Delta H_{vap} \quad (3)$$

The heat loss from the vessel surface (Q_{loss}) was evaluated through the global heat transfer coefficient of the system, obtained from the decrease of temperature (0.36 ºC/min) over time of a hot sample after finishing the ultrasound treatment.

To estimate the total energy consumed by the ultrasound equipment, power consumption ($P_{consumed}$) was recorded during the pretreatments.

The ratio of energy absorbed by the sample to energy consumed by the ultrasound equipment was referred to as energy efficiency, and was calculated as follows (Eq.4):

$$Efficiency\ (\%) = \frac{E_{absorbed}}{\int P_{consumed}\,dt} \quad (4)$$

2.4. Thermal treatment

The thermal history (temperature versus time) of an ultrasound pretreatment at 100% amplitude and 70ºC was repeated in a conventional thermal treatment process using an electric heater, and it was followed by a conventional extraction stage. The comparison of both pretreatments allowed for the investigation of a probable non-thermal effect of ultrasounds.

2.5. Total phenol content analysis

Total phenol content (TPC) was chosen to quantify polyphenols extraction kinetics. TPC is an indicative parameter when obtaining valuable compounds from grape marc, but does not provide relevant information on antioxidant activity or the content of any specific component of interest. However, its ease of analytical measurement and its representative behavior in global polyphenol extraction makes of TPC a suitable election to follow the extraction kinetics in this process. Ghafoor et al (*2009*) found that up to 60ºC during 30 min TPC, antioxidant activity and anthocyanin content change similarly in an UAE process of grape seeds.

TPC was quantified, using the Folin-Ciocalteu method, as Gallic acid equivalents per gram of dry material [mg GA/g DM]. (*Singleton et al. 1999*) A volume of 40 µl of filtered sample (0.2 µm, nylon membrane) was diluted with distilled water (3 ml) and mixed with 200µl of Folin-Ciocalteu reagent. After 10 minutes saturated Na_2CO_3 was added to the solutions, and the samples were incubated at 40ºC for 30 min. Absorbance was measured at 765 nm (UV 2550

Shimadzu UV/VIS spectrometer) and known concentrations of diluted Gallic acid were used for calibration.

2.6. Extraction kinetics and activation energy

The extraction yield obtained from TPC measurements was plotted versus extraction time and first order kinetics was fitted to the experimental points (Eq.5)

$$TPC=A\cdot(1-exp(-\beta t)) \quad (5)$$

The extraction process is characterized by 1) the final extraction yield, A [mg GA/g DM], and 2) the initial extraction rate, $A\beta$ [mg GA/g DM min], where β is the time constant [min^{-1}]. The fitting of the experimental data was achieved by absolute error minimization, using the Solver tool in MS Excel.

The dependence of the extraction rate constant on temperature can be described by the Arrhenius equation (Eq. 6):

$$\beta=\beta_0\cdot exp(-E_a/RT) \quad (6)$$

, where β_0 is the pre-exponential temperature independent factor [min^{-1}], R is the universal gas constant (8.314 J/mol K), T is the absolute temperature [K] and E_a is the activation energy of extraction [J/mol]. These parameters, E_a and β_0, were obtained from the slope and interception in the linear fitting of $ln(\beta)$ versus $1/T$, respectively.

2.7. Data Analysis

Full factorial experimental design (3^2) was used to study the ultrasound effect in extraction experiments. Two-way ANOVA analysis was performed where results were considered to be significantly different at 95.0 % confidence level. Dependent variables were the final extraction yield (mg TPC/g DM) and the initial extraction rate. Power level (50-75-100%) and Absorbed energies corresponding to temperatures (40-55-70 ºC) were the two factors, each on 3 levels.

3. RESULTS AND DISCUSSION

3.1. Absorbed energy in US and thermal pretreatments

The calorimetric evaluation of the absorbed acoustic energy showed efficiencies between 32% and 46% (Table 1) depending on operating conditions. At 100% amplitude efficiency was lower, (35.0 ± 2.6%) compared to amplitudes of 75% and 50%, where 41.8±3.6% and 40.7±3.8% efficiencies were measured, respectively. When high power intensity is applied, poor coupling may occur between the US probe and the medium because of the cloud formation of cavitation bubbles on the horn tip. (*Ratoarinoro et al. 1995*) Relatively low efficiencies were obtained because the liquid height in experimental setup was not optimized, but fixed to provide identical conditions in energy absorption. The nature and height of the liquid under US influences the procedure efficiency (*Romdhane et al. 1994, Ratoarinoro et al. 1995*).

Table 1 *Evaluation of absorbed energy under different US pre-treatments*

Amplitude	Temperature	time $_{pretreatment}$	Delta T	Evaporation	Q $_{sensible}$	Q $_{latent}$	Energy $_{absorbed}$	Energy $_{emitted}$	Efficiency
%	ºC	min	ºC	g	kJ	kJ	kJ	kJ	%
Control	40	0	-	-	-	-	-	-	-
Control	40	0	-	-	-	-	-	-	-
100	40	3	16	0.9	9.02	0.49	9.50	29.56	32%
100	55	5.5	31	0.8	17.47	0.43	17.90	49.73	36%
100	70	9	46	2.1	25.92	1.13	27.06	72.36	37%
75	40	3.5	17	1.0	9.58	0.54	10.12	24.20	42%
75	55	7	31	1.5	17.47	0.81	18.28	42.65	43%
75	55	7	30	1.6	16.91	0.86	17.77	42.06	42%
75	55	6	30	1.6	16.91	0.86	17.77	38.27	46%
75	70	14.5	45	3.9	25.36	2.11	27.46	75.47	36%
50	40	5.5	15	1.8	8.45	0.95	9.40	24.22	39%
50	55	7.5	31	1.7	17.47	0.92	18.39	41.21	45%
50	70	22	43	4.2	24.23	2.27	26.50	70.49	38%
Thermal	70	10	46	-	25.92		25.92	-	-

The largest contribution to the calculated absorbed energy corresponded to sensible heat, because the pretreatment time was always set to achieve a final temperature preset value, and no holding times followed. At lower temperatures (40, 55ºC) the sensible heat accounted for 95% of the total absorbed energy, while in the case of 70ºC it was reduced to 92% due to the more intense evaporation of the solvent. Lower sensible heat contribution could also be observed when the amplitude was lowered to 50%, as longer treatment times also favor solvent evaporation.

Heat loss from the extraction vessel (Q_{loss}) was negligible (less than 1 %) in all cases, demonstrating the sufficient thermal insulation of the experimental setup. This contribution has not been finally considered in the calculation of the absorbed energy. To achieve comparable

conditions at different power intensities (amplitude levels) and correlate them with the amount of absorbed energy amount, it is necessary to avoid heat loss in the experiments, as it may lengthen the treatment times in different proportion for different power intensities. Excessive evaporation would change the solvent composition and concentration of the active compounds, and may alter the analytical measurement results.

In the case of conventional thermal pretreatment at 70ºC, the efficiency of the equipment was not evaluated, however, the absorbed energy was determined from sensible heat. This experimental setup was closed, so evaporation can be excluded, and heat loss from the surface was unlikely as a round-shaped external heat source was applied to the round-shaped glass container. This value is also reported in Table 1.

3.2. Comparison of extraction kinetics of US pretreatments

In control extractions, where no pretreatment was applied, the first order kinetics fitted well to the experimental values (Figure 1) and good reproducibility was achieved with parallel experimental setups. The maximum value of the total phenol content was reached before the third hour of extraction in control experiments, and also in the case of some pretreated samples. However, in most of the US extractions with pretreatment, the extraction yield was still increasing during the third hour, although with lower extraction kinetics compared to the initial one. This phenomenon has also been observed elsewhere (*Virot et al. 2010*). The irregular geometry and structure of grape marcs may explain (*Cárcel et al. 2010*) the difficulties in completing the extraction.

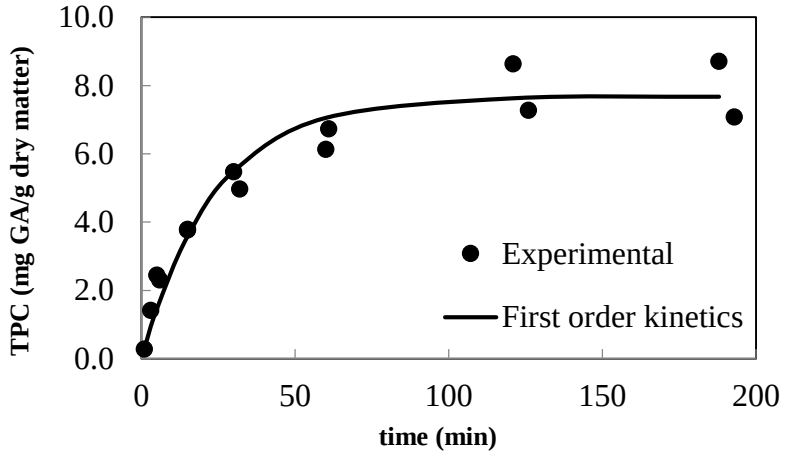

Figure 1 *Extraction kinetics in control experiments in duplicate*

Total phenol yield just after US pretreatment, but before conventional extraction, was also measured and reported in Figure 2 as a function of the energy absorbed in the pretreatment. The amount of polyphenols that are extracted during the pretreatment increases sharply with increasing absorbed energy. Palma and Barroso (*2002*) found that the active compounds of wine making by-products reached the highest yield at 70ºC in UAE and decreasing was observed over this temperature. This variable was the most significant in antioxidant extraction from red grape jam in US bath (*Morelli and Prado 2012*). Comparing the different amplitudes with the same absorbed energy, lower amplitude levels gave a slightly higher but not significant total phenol yield, which was due rather to the longer treatment time than to changes in power intensity.

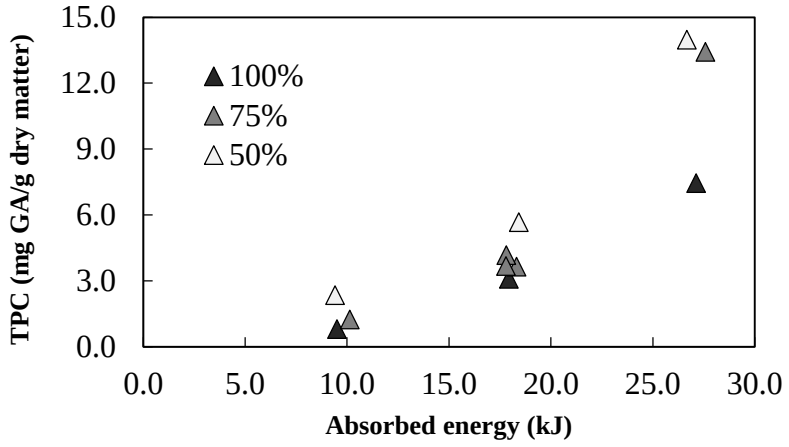

Figure 2 *TPC yields after US pre-treatments, before conventional extraction at 40-55-70ºC and amplitude levels of 50-75-100%.*

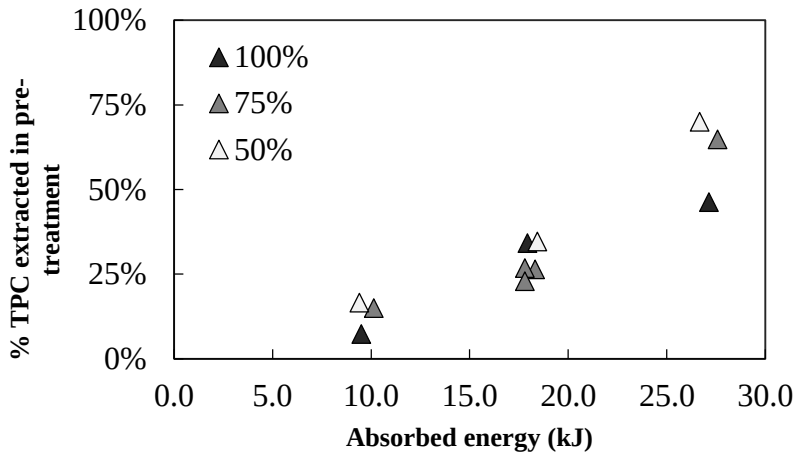

Figure 3 *TPC yields after US pre-treatment, shown as the percentage of the total TPC yield of the extraction process versus the absorbed energy of the pre-treatments.*

Figure 3 shows the percentage of TPC extracted just after the pretreatment, before conventional extraction, versus the US energy absorbed in the pretreatment. Up to 70 percent of the final yield may be achieved during this stage. This maximum value was obtained at 70 °C using the lowest amplitude level (50%). In general, the 50% amplitude resulted in higher percentages at all temperatures; however, the final TPC yield was not so distinct using different amplitudes. This result shows how the pretreatment, without subsequent extraction stage, may be effective enough to extract the same or even higher amount of antioxidants from grape marc than the conventional process.

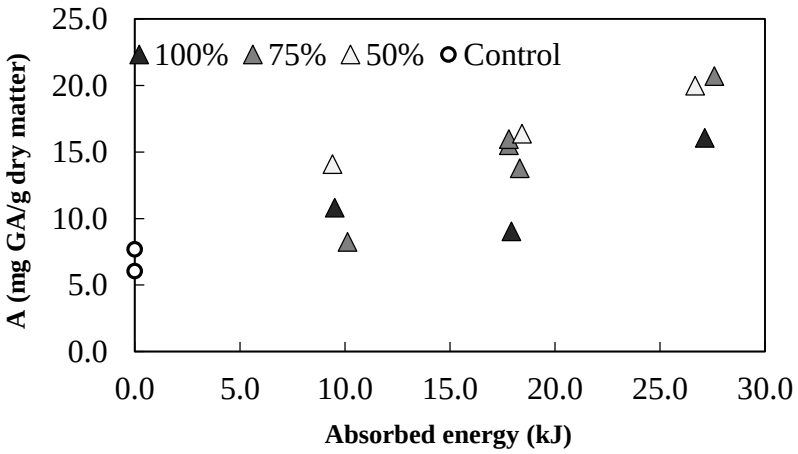

Figure 4 *Final yields in the extraction process*

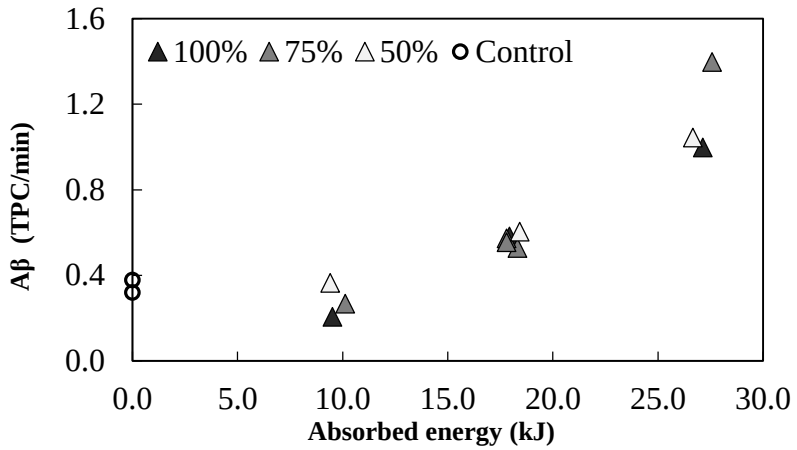

Figure 5 *Initial extraction rate (Aβ) in the extraction process*

US pretreatments were followed by a conventional stirred extraction at 40ºC for 3 hours. Significant increases in the extraction stage were achieved both, in terms of extraction yields (Figure 4) and of initial extraction rates (Figure 5). These increases were higher with increasing temperature at all applied amplitude levels, when compared to the control extractions. The final yield (*A*) increased with decreasing amplitude from 100% to 50%: 10.8 to 14.1 at 40ºC, 9.0 to 16.4 at 55ºC and 16.0 to 20.0 at 70ºC. However, the statistical analysis showed a significant but not relevant effect of amplitude on initial extraction rate. The main improvement in extraction kinetics was related to the amount of energy absorbed during the pretreatment and not to the intensity of this energy input (amplitude).

3.3. Activation energy

The Arrhenius equation was used to determine the activation energy (E_a) of the extraction process. Figure 6 represents the relationship between the time constant (β) and temperature (T) according to Eq (6). From the slope of the linear correlation E_a=25.8 kJ/mol and β_o = 2.97 10^4 min^{-1} were obtained. A similar activation energy value was obtained for E_a in the case of oil extraction from jatropha seeds. (*Amin et al. 2010*) A lower value (E_a=14.5 kJ/mol) was obtained in the case of polyphenol extraction from pomegranate marc (*Qu et al. 2010*). In the study mentioned, milled samples were used for the extraction process, facilitating mass transfer. Milling was also proposed as a necessary pretreatment for UAE of antioxidants from rosemary leaves (*Rodriguez-Rojo et al. 2012*)

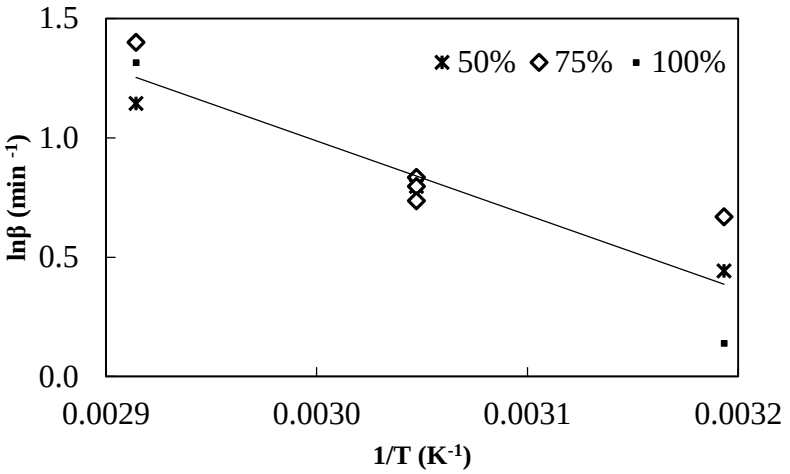

Figure 6 *Linear regression of the Arrhenius equation*

3.4. Non-thermal effect of US pretreatment on extraction kinetics

It is difficult to distinguish different types of energy that are provided to the samples by ultrasounds. The absorbed energy of the ultrasound is finally converted into heat, and this temperature increment itself may already change the extraction rate and final yield, hiding the structure disruption caused by the mechanical energy of sonication.

Table 2 *Experimental conditions for non-thermal US effect study.*

Amplitude	Temperature	time	$A_{after\ US}$	A_{final}	β	Aβ	$E_{emitted}$
%	ºC	min	mg GA/g DM		min^{-1}	TPC/min	kJ
100	70	9	7.44	16.06	0.062	1.00	72.36
100 (cooled)	45	9	3.18	14.26	0.028	0.40	86.94
Thermal	70	10	9.80	17.17	0.060	1.04	-

To study the separated thermal and non-thermal effect of US, three types of experiments were compared (Table 2): 1) 100% amplitude was applied for 9 minutes with thermal insulation around the extraction vessel. The absorbed acoustic energy directly heated the sample, and temperature increased up to 70ºC. 2) 100% amplitude for 9 minutes with water cooling through the jacketed extraction vessel. In this case, part of the US absorbed energy was removed as thermal energy by the circulating cooling medium. The final temperature was only 45ºC. 3) Conventional heating was used to heat up the sample up to 70ºC, over the same time period as in 1) and 2). After these pretreatments conventional extraction was performed in all cases for 3 hours long at 40ºC, and extraction kinetics were measured.

Results from 1) and 2) make it possible to compare the same US energy at different temperatures, while 1) and 3) compare the same amount of absorbed energy from different sources at the same temperature. On Figure 7, TPC values are presented along the extraction after the pretreatments.

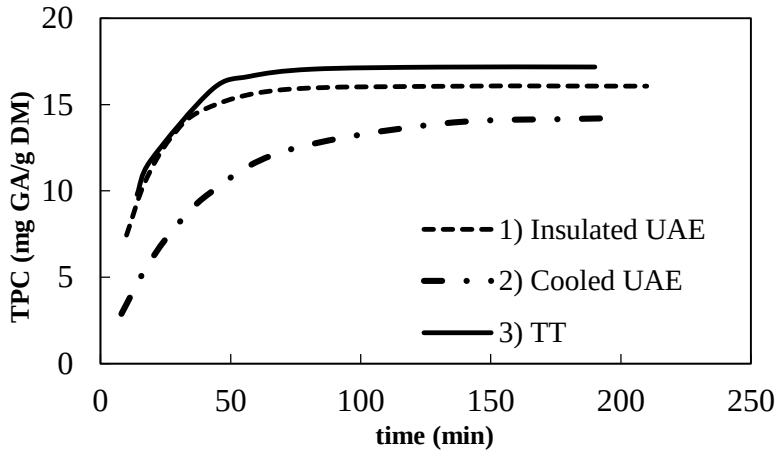

Figure 7 *Extraction kinetics after pre-treatment: 1) Insulated UAE at 70ºC, 2) cooled UAE using the same energy input, and conventional thermal pre-treatment (TT) at 70ºC.*

Pretreatments 1) and 3) followed similar extraction kinetics regardless of the applied energy source (thermal or US). By contrast, with the cooled US treatment a 60% lower initial rate was observed than in 1), although similar final extraction yields were reached: 14.26 and 16.06 (mg GA/g dry matter) for cooled and insulated experiments, respectively. These results indicate that only a thermal effect can be observed in terms of total phenol extraction yield and rate, and there is no clear evidence of a specific US effect apart from the thermal one.

4. CONCLUSIONS

The effect of the energy absorbed in the US pretreatment of grape marc for polyphenol extraction was studied. Energy efficiency of the equipment was between 32% and 46%. The majority of the absorbed energy (≈ 95%) increased the temperature in the extraction vessel, while the rest caused evaporation of small amounts of solvent.

First order kinetics was suitable to correlate the extraction results. The final yield of phenolic compounds and initial extraction rate increased significantly at increasing temperatures, while the intensity of the absorbed energy (amplitude) had little effect on extraction kinetics. The pretreatment itself was effective enough to extract up to 70% of the final total phenol content, which significantly shortens the process, and further increases production yield.

The activation energy of the process was determined as (E_a=25.8 kJ/mol), using the Arrhenius equation.

The existence of a possible non-thermal effect of US was not detected when comparing US at 70ºC with a conventional thermal treatment. The single thermal effect was confirmed by comparison of US treatments with the same amplitude and treatment time at different temperatures.

NOMENCLATURE:

Abbreviations:

US	*ultrasound*
UAE	*ultrasound assisted extraction*
GA	*Gallic acid*
DM	*Dry matter*
TPC	*Total phenol Content*

Parameters:

D	*diameter*	*mm*
$P_{consumed}$	*consumed power*	*W*
$E_{absorbed}$	*absorbed energy*	*kJ*
$Q_{sensible}$	*sensible heat*	*kJ*
Q_{latent}	*latent heat*	*kJ*
Q_{loss}	*heat loss*	*kJ*
m_{sample}	*grape and solvent weight*	*g*
ΔT	*temperature increase*	*ºC or K*
c_p	*specific heat capacity*	*kJ/kgK*
m_{vap}	*evaporated solvent weight*	*g*
ΔH_{vap}	*heat of evaporation*	*kJ/kg*
A	*extraction yield*	*mg GA/g DM*
β	*time constant*	min^{-1}
$A\beta$	*initial extraction rate*	*mg GA/g DM min*
β_0	*pre-exponential temperature independent factor*	min^{-1}
R	*universal gas constant*	*8.314 J/molK*
T	*absolute temperature*	*K*
E_a	*energy of activation*	*J/mol*

ACKNOWLEDGEMENTS

The authors thank the Spanish Ministry of Economy and Competitiveness for the Project CTQ2010-15475 and the project of ENE2012-33613. Katalin Sólyom thanks the University of Valladolid for the financial support provided by the "FPI UVa" scholarship.

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Chapter 6.

Thermal degradation of grape marc polyphenols

THERMAL DEGRADATION OF GRAPE MARC POLYPHENOLS

Abstract

Bioactive compounds of wine making by-products are of interest in food, cosmetics and pharmaceutical industries. Extraction of antioxidants under mild conditions is time-consuming, giving ground to the development of intensification processes where operation at high temperature may deteriorate extract quality. This study examined thermal degradation of solid grape marc and liquid grape marc extract (80-100-150 °C). The decrease in anthocyanin content was modelled under non-isothermal conditions by first order kinetics, using the Arrhenius equation. Simulated degradation under isothermal heating showed that the solid grape marc is more sensitive by one order of magnitude to heat than the liquid extract. Despite the initial increment in total phenol content and antioxidant activity, analyses confirmed the vulnerability of solid grape marc. It is suggested that an optimal combination of temperature, treatment time and also raw material environment could be found in process intensification.

1. INTRODUCTION

Worldwide wine production generates a large amount of semi-solid residue (grape marc), with high antioxidants content. Flavonoids, anthocyanins and stilbene derivatives are bioactive compounds present in this residue, and they are of interest for food-, cosmetics- and pharmaceutical industries due to their antioxidant, antimicrobial, antiviral or anticarcinogenic features (*Nassiri Asl and Hosseinzadech, 2009*). The elimination of these compounds is also desirable before using the grape pomace as fertilizer, in order to avoid inhibition problems (*Negro et al, 2003, Pinelo et al, 2006*). Mild extraction techniques (maceration and stirred extraction) are time consuming, and usually require an excessive amount of solvent, leading to an inefficient and costly recovery process (*Spigno and De Faveri, 2007, Spigno et al, 2007, Lafka et al, 2007, Amendola et al, 2010*).

The proposal of novel techniques and pre-treatments in order to enhance the extraction kinetics of valuable components may also imply high temperature during processing. Extraction at high pressure and temperature (110 °C) for 60 minutes was applied and compared to microwave assisted extraction for better recovery of bioactive compounds from grape waste (*Casazza et al, 2010*). Peralbo-Molina and co-workers (*2012*) used superheated extraction at 180 °C for 60 min in competition with microwave and ultrasound assisted extraction to enhance the extract quality and process efficiency, compared to conventional maceration. Drying pre-treatment was also applied in the case of grape stalks between 40 and 115 °C, and improved extraction kinetics were described by a diffusion model (*Garcia-Perez et al 2010*).

Although these process intensification methods increased the quantity or the quality of the extract, their destructive effect on the antioxidant content may be masked by a larger beneficial effect on the extraction stage efficiency. The physical complexity of the natural raw material and the variety of experimental conditions may modify interaction between active compounds, thus changing the final product quality. The effect of thermal treatments on liquid extracts was elsewhere studied (*Wang and Xu, 2007, Cruz et al, 2007, Cisse et al, 2009*), and first order kinetics degradation was found for anthocyanins, while for other antioxidants this tendency was not so clear. Drying of raw material deteriorated the antioxidant quality of grape peels when treated above 60°C (*Larrauri et al, 1997*).

Heating of grape seeds prior to extraction released more polyphenols, verified by antioxidant activity and HPLC analysis (*Kim et al, 2006*). Anthocyanin content of dry bilberry extracts was reduced after 30 min, 30% in dry bilberry extracts at 100 °C, while antioxidant activity

increased with increasing temperature (*Yue and Xu 2008*). It is clear that the raw materials variability and the different heating conditions are hardly comparable in terms of temperature and time, solid content, or the presence of extraction solvent. Consequently, the aim of this study was to compare the effects of heating on the semi-solid grape marc waste and on its liquid extract. Heating was performed at 80-100-150 °C up to for 4 hours in order to study degradation kinetics in terms of total phenol content, antioxidant activity and anthocyanin content. Kinetic parameters of anthocyanin degradation were obtained by regression of non-isothermal heating experimental values to a first order kinetics model. These parameters were later used to estimate and compare degradation in isothermal heating of semi-solid grape marc and its liquid extract.

2. MATERIALS AND METHODS

2.1. Grape marc

A mixture of red grape seeds and skins (grape marc) of Tempranillo grape variety from the region of Toro (Spain) was used in the experiments. This residue was generated in the wine making process, after long maceration with post-fermentative phase. The grape marc was distributed in 100 g packages and stored at -18 °C till the experiments to conserve the initial composition of the raw material and avoid deterioration by the time. Defrosting was performed at 4 °C overnight.

2.2. Heat treatment of grape marc

In these experiments, the raw material was first exposed to heat, and then further extracted with solvent. Grape marc (10±0.001 g) was filled in three identical stainless steel tubes (length: 220 mm, inner diameter: 12.3 mm), and closed with stainless steel stopper to hold up the arising pressure during the experiment. The tubes were placed in an oven, set at 80-100-150 °C, and were removed consecutively after 1, 2 and 4 hours for analysis. The series at 150 °C was repeated with sampling times at 20-40-60 minutes to better follow the fast degradation observed. Tubes were immediately cooled down in an ice bath and further stored at 4 °C until extraction. Also, one fraction of original grape marc was kept for extraction, without previous heat treatment, to use it as a control sample in comparison to the heated material. Repeatability of the treatment with different grape marc batches was determined by repeating the full experiment at 150 °C. The variability between the tubes was determined by removing three tubes after 1 hour at 150 °C, and further analysing them.

After the heat treatment, grape marc was removed from the tubes and placed in glass vessels for extraction with 50 ml of 50% volumetric mixture of ethanol (96%) and acidified water (pH=1). Extraction was performed for 1 hour at 40 °C, under continuous stirring on an orbital shaker. Extraction was stopped by separating liquid and solid phases in centrifuge (4000 rpm, 5 min). The supernatants were filtered (PET membrane, 0.2 µm) for further analyses.

2.3. Heat treatment of filtered extract

In the second series of experiments, extraction was first performed from the grape marc, and the obtained extract was further exposed to heat treatment. An amount of 40 g of raw material was extracted in 200 ml 50% volumetric mixture of ethanol (96%) and acidified water (pH=1; H2SO4) at 40 °C for 24 hours in the dark. The aim of this sample was to study the effect of temperature on polyphenols stability in the absence of solid phase. Beacause of this reason, a longer extraction time (24 h) was chosen to obtain high extraction yield, so that most of the polyphenols originally present in the solid sample could be extracted to the liquid sample before the following heat treatment was performed. In the case of the extraction of the preheated grape marc, only 1 hour extraction time was found to be enough to evaluate the differences after the heat treatments. After the extraction, centrifuge was used for phase separation (4000 rpm, 5 min) and the supernatant was filtered through a 0.45 µm borosilicate filter (Millipore). The obtained filtered extract was filled in five stainless steel tubes (length: 160 mm, inner diameter: 3.9 mm). In this case thinner tubes were used to achieve faster heating of the filtered extract. In the case of the grape marc, larger inner tube diameter was necessary to fit the skin and seed particles in the tube. Closed tubes were placed in the oven at 80-100-150 °C, and removed one by one after 20-40-60-120-240 minutes. After immediate cooling in ice bath, the extracts were filtered (PET membrane, 0.2 µm) and kept refrigerated for further analysis. A fraction of the original unheated filtered extract was also kept as control sample for comparison**.**

2.4. Temperature evolution in heat treatments

Thermal history of the experimental setup was followed by temperature measurement inside separate tubes with a K-type thermocouple. Temperature was recorded until the set value was reached.

2.5. Analysis

The ***moisture content*** of the grape marc (≈ 85 w/w %) was determined in triplicate for every used grape marc batch by weight difference after drying at 105ºC for 24 hours, until constant weight

Total phenol content (TPC) was quantified using the Folin-Ciocalteu method, as Gallic acid equivalents per gram of dry material [mg GA/g DM] [17]. A volume of 40 µl of filtered sample was diluted with distilled water (3 ml) and mixed with 200 µl of Folin-Ciocalteu reagent. After 10 minutes, saturated Na_2CO_3 was added to the solutions, and the samples were incubated at 40ºC for 30 min. Absorbance was measured at 765 nm (UV 2550 Shimadzu UV/VIS spectrometer) and known concentrations of diluted Gallic acid were used for calibration.

Total monomeric anthocyanin pigment content was determined by the pH differential method [18]. Results are expressed on the basis of cyanidin-3-glucoside [mg cyanidin-3-glucoside/ g dry matter].

Antioxidant activity was measured in terms of ***Oxygen Radical Absorbance Capacity*** (ORAC) based on the Application note 148 (BMG-Labtech). 96 well-plate was used in Fluostar Optima (BMG-Labtech) to determine the signal quench from fluorescent probe (100 nM Fluorescein in PBS) due to the reactive oxygen species, generated from the thermal decomposition of 240 mM AAPH [2,2`-azobis(2-methylpropionamidine) dihydrochloride] at 37°C. Trolox (12.5 – 200 µM) standard (3 fold), or diluted (1:100) grape marc extract (6 fold) were added in the wells. Depending on the antioxidant activity, the stability of the fluorescence signal was affected. Fluorescence was recorded over time, and from area integration under the kinetic curves in every well, the antioxidant capacity was estimated (Optima-MARS Data Analysis). ORAC values of the extracts were compared to Trolox standards and expressed as Trolox equivalents (TE) [µmol Trolox/ g dry material].

2.6. Experimental design

Heat treatment on grape marc was performed from different packages of 100 g. Every day a new package was opened, from which the corresponding moisture content determination was performed and control sample was extracted. Repeatability between the different batches was studied by control samples and a heat treatment in triplicate at 150°C with 3 sample tubes withdrawn after 60-120-240 minutes, respectively. Variation between the different tubes was determined from one grape marc batch by exposing the 3 tubes to 150°C for 60 minutes heat treatment.

Heat treatment on filtered extract was performed from one extraction batch. Repeatability was studied by a heat treatment at 150°C for 30 minutes in triplicate. All analytical procedures were used on the repeated treatments. The estimated errors from the repeated experiments were considered for the other experimental conditions as well. An overview of the performed experiments is presented in Table 1.

Table 1 Experimental design of thermal treatments

Sample		Temperature (°C)	Time (min)
Grape marc (different batches)	1	150	0; 60; 60; 60
	2	150	0; 60;120;240
	3	150	0; 60;120;240
	4	80	0; 60;120;240
	5	100	0; 60;120;240
	6	150	0; 20;40
Filtered extract (Same batch)	7	80	0; 20; 40; 60; 120; 240
	8	100	0; 20; 40; 60; 120; 240
	9	150	0; 20; 40; 60; 120; 240
	10	150	0; 30; 30; 30

3. RESULTS AND DISCUSSION

3.1. Repeatability

The analyses of the different grape marc batches after 1 hour of extraction without heat treatment (Table 1, samples 1-6.) resulted in 4.01 ± 0.57 mg cyanidin-3-glucoside/ g DM, 44.81 ± 10.48 mg GA/g DM, and 426.9 ± 115.1 µmol Trolox/ g DM for anthocyanin, total phenol and ORAC values respectively. Errors determined from the standard deviation of triplicated analyses from the same batch with the same treatment (Table 1, sample 1) resulted 9.5%, 1.6% and 13.0% for anthocyanin, total phenol and antioxidant values respectively. These errors allow the comparison between results of the same batch to study the behaviour of the kinetics during the treatment, however, because of sample variability, separated batches would not provide exact comparable results. For this reason, along the following discussion on the obtained results, experimental values are compared to their corresponding control value from the same grape marc package.

In the case of the filtered extract (Table 1, samples 7-9) untreated extracts were compared from the same batch on three days in a row. Anthocyanin content was 3.81 ± 0.10 mg cyanidin-3-glucoside/ g DM, total phenol content was 82.79 ± 2.67 mg GA/g DM and ORAC value was 470.0 ± 62.5 µmol Trolox/ g DM. Triplicated experiment on filtered extract with the same heat treatment (Table 1, sample 10) resulted in 16%, 1.1% and 13.3% of error for anthocyanin, total phenol and antioxidant values, respectively. As in the case of the thermal treatment on grape marc, kinetic studies of anthocyanin degradation in filtered extract were presented as relative concentration values (Section 3.2.).

3.2. Anthocyanin content

From the experimental values obtained in the case of anthocyanin degradation (Figure 2 a, b) first order kinetics can be proposed to describe the concentration evolution (*dC/dt*) of monomeric anthocyanins over the heating process (Eq 1).

$$\frac{dC}{dt} = -BC \qquad (1)$$

The rate parameter (*B*) depends on temperature according to the Arrhenius model (Eq 2), as it was already suggested by others (*Wang and Xu 2007, Cisse et al, 2009, Patras et al 2010*).

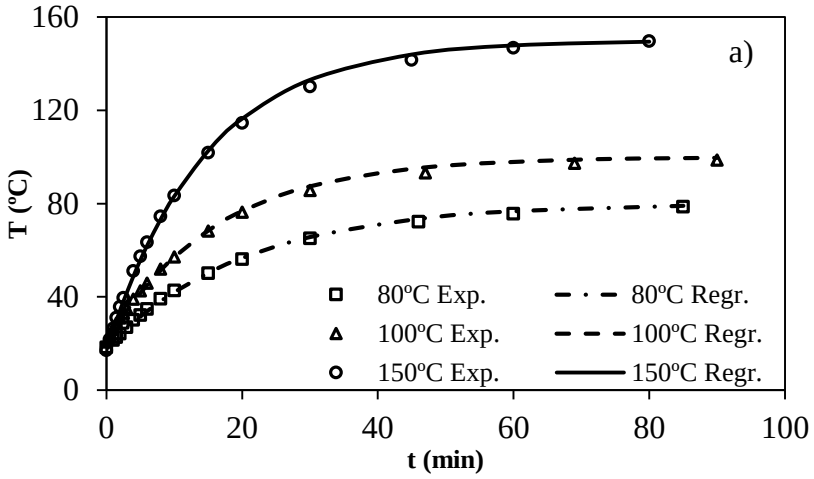
$$B = B_0 \cdot e^{-\frac{E_a}{RT}} \quad (2)$$

The heating of the sample inside of the tubes was slow, and a period between 30 and 60 min was required to reach the temperature value set in the oven. Since the rate parameter (*B*) is temperature dependent, it was necessary to correlate temperature evolution in the heating process as a function of time to integrate Eq. 1. In previous papers (*Patras et al, 2010, Dolan 2003, Mishra et al, 2008*), this effect has been modelled using the "*time-temperature history (β)*", a scaling factor of the rate constant obtained by integration of the rate parameter over time.

In this paper, the heating temperature evolution of the samples was found to be successfully correlated by first order kinetics (Eq 3):

$$T(t) = T_{initial} + (T_{final} - T_{initial})(1 - e^{-k_T t}) \quad (3)$$

The value of the heating rate constant (k_T) was obtained for each experiment by minimizing the sum of the squares of the differences between experimental and calculated temperatures. Experimental values and regressed functions are shown in Fig. 1 and correlation parameters in Table 2.



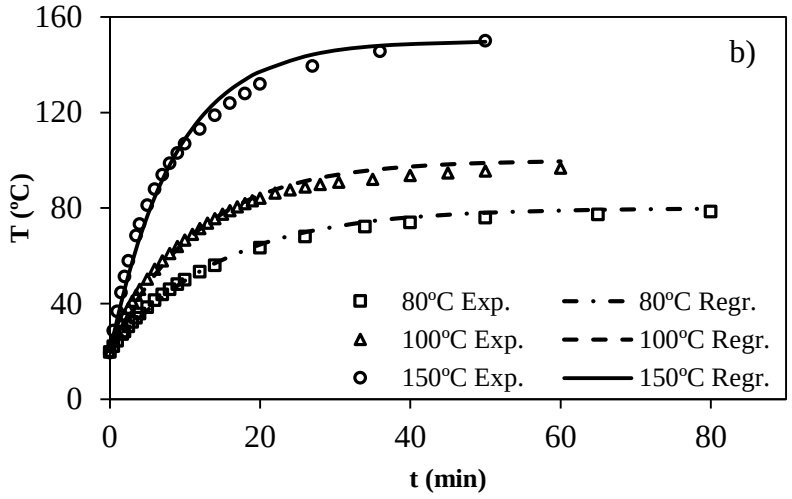


Figure 1 *Experimental (Exp.) and model fit (Regr.) temperature values of the non-isothermal heating of a) grape marc and b) filtered extract*

Table 2 *Correlation parameters of the heating process and the anthocyanin degradation kinetics*

Parameter		**Solid**	**Liquid**
$k_{80ºC}$	min^{-1}	0.0486 ± 0.0006	0.0688 ± 0.0014
$k_{100ºC}$	min^{-1}	0.0615 ± 0.0010	0.0851 ± 0.0015
$k_{150ºC}$	min^{-1}	0.0688 ± 0.0009	0.1156 ± 0.0030
B_0	min^{-1}	$1.67*10^6 \pm 0.34*10^6$	$1.32*10^5 \pm 1.62*10^4$
E_a	*J/mol*	55980 ± 620	53196 ± 399

By combining Eqs 1 to 3, anythocyanin degradation can be described (Eq. 4) by the values of the pre-exponential constant of the degradation rate parameter (B_0), the degradation energy of activation (E_a) and the heating rate constant of the thermal treatment(k_T) :

$$\frac{dC}{dt} = -B_0 \cdot \exp\left(-\frac{E_a}{R \cdot (T_{initial} + (T_{final} - T_{initial})(1 - e^{-k_T t})}\right) \cdot C \qquad (4)$$

This differential equation was integrated by the Euler's method, with a time step $\Delta t = 0.1$ min.

A single pair of values (B_0, E_a) is used to describe all degradation experiments with the same material (grape marc or liquid extract), regardless of the heating process or temperature. These values were obtained by minimizing the following objective function (Eq. 5):

$$\chi^2 = \sum_{j=1}^{s}\sum_{i=1}^{n_j}\left(C_{ij}^{\exp} - C_{ij}^{cal}\right)^2 \quad (5)$$

, where $C_{ij}^{\exp}$ and C_{ij}^{cal} are the experimental and calculated values of anthocyanin concentration, n_j the number of data points in degradation experiment j, and s the total number of experiments with the same material (grape marc or liquid extract).

To locate initial estimates for B_0 and E_a, fixed values of B_0 were established in a range of 10^4 – 10^7 min^{-1}, and corresponding E_a values were calculated by minimizing the objective function (χ^2). The pair (B_0, E_a) providing the minimum value of χ^2 was used as initial estimate for the simultaneous minimization of both parameters.

The standard error of the correlation parameters (B_0, E_a) was calculated (Eq. 6):

$$\sigma_{a_k}^2 = \frac{\chi^2}{N-p}\left[\sum_{i=1}^{N}\left(\frac{\partial C_i}{\partial a_k}\right)^2\right]^{-1} \quad (6)$$

, where N is the total number of data points $N = \sum_{j=1}^{s} n_j$, p the number of correlation parameters, and a_k the corresponding correlation parameter (B_0 or E_a).

Considering the wide sample variability and that a single set of parameters is used at each temperature for all experiments, the correlation results show an acceptable agreement between experimental and correlated values (Figure 2 a, b): R^2= 0.9261 in the case of the grape marc and R^2= 0.9731 in the case of the filtered extract. The graphs initially show a short flat region, corresponding to the heating stage, where temperature was not high enough to produce significant anthocyanin degradation. After 10 or 20 min, degradation begins and increases rapidly due to the combination of temperature rise and first order degradation kinetics.

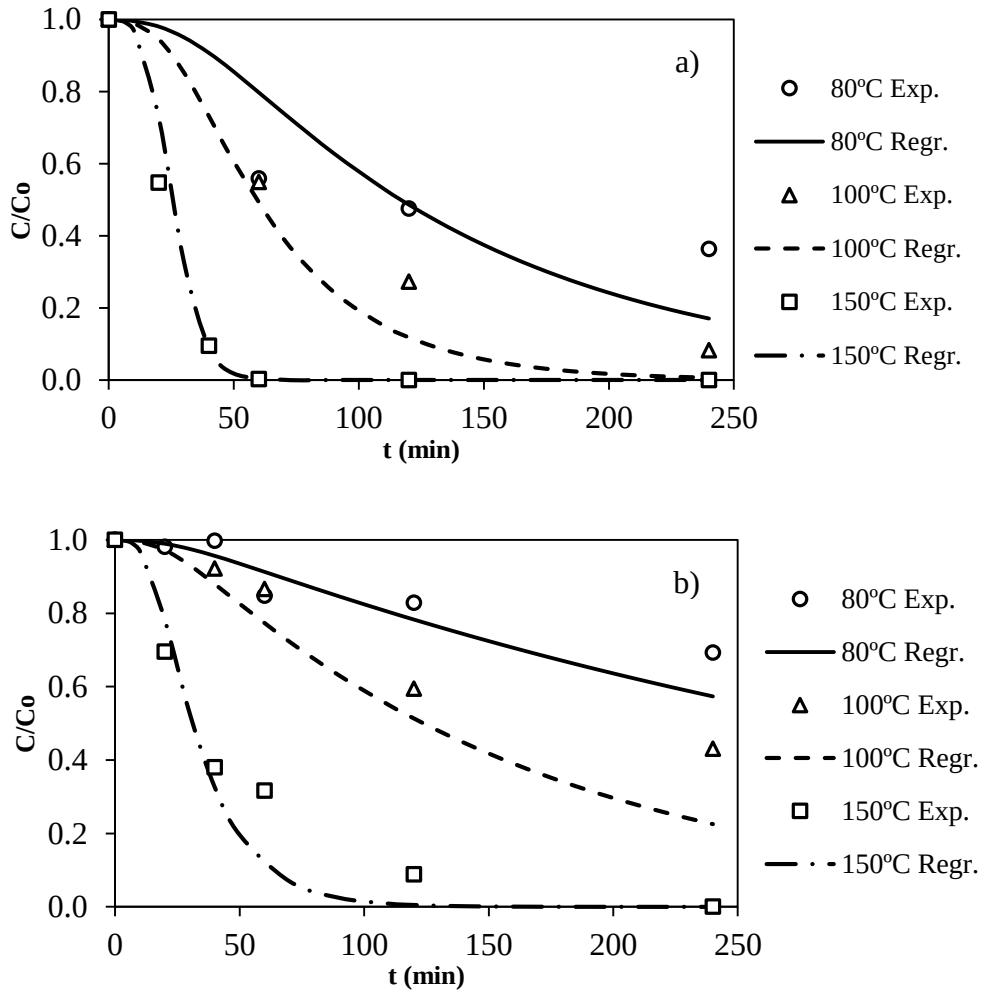


Figure 2 *Experimental (Exp.) and model fit (Regr.) values of the non-isothermal degradation kinetics of anthocyanin content of a) grape marc and b) filtered extract at 80-100-150 °C*

The activation energy for the grape marc (Table 2) is slightly higher than the one for the filtered extract, which suggest that temperature rise will degrade anthocyanins in grape marc samples more rapidly. The pre-exponential term is higher by one order of magnitude for the degradation of anthocyanins in the presence of grape marc than in the isolated filtered extract. This fact shows a higher tendency for reactions converting monomer anthocyanin in other molecules when the solid is present. Precise reaction mechanisms are difficult to describe in complex, natural raw materials, where numerous possibilities and molecules may play an important role. However, the activation energies values are in agreement with those from other studies (*Cisse et al 2009, Yue and Xu 2008, Patras et al, 2010*).

Isothermal heating degradation can be predicted at any temperature (Figure 3 a, b) from the so obtained correlation parameters. The anthocyanin concentration of the filtered extract reduces to 50% after 13 minutes at 150 °C, while 4.5 hours are required for the same reduction ratio at 80 °C.

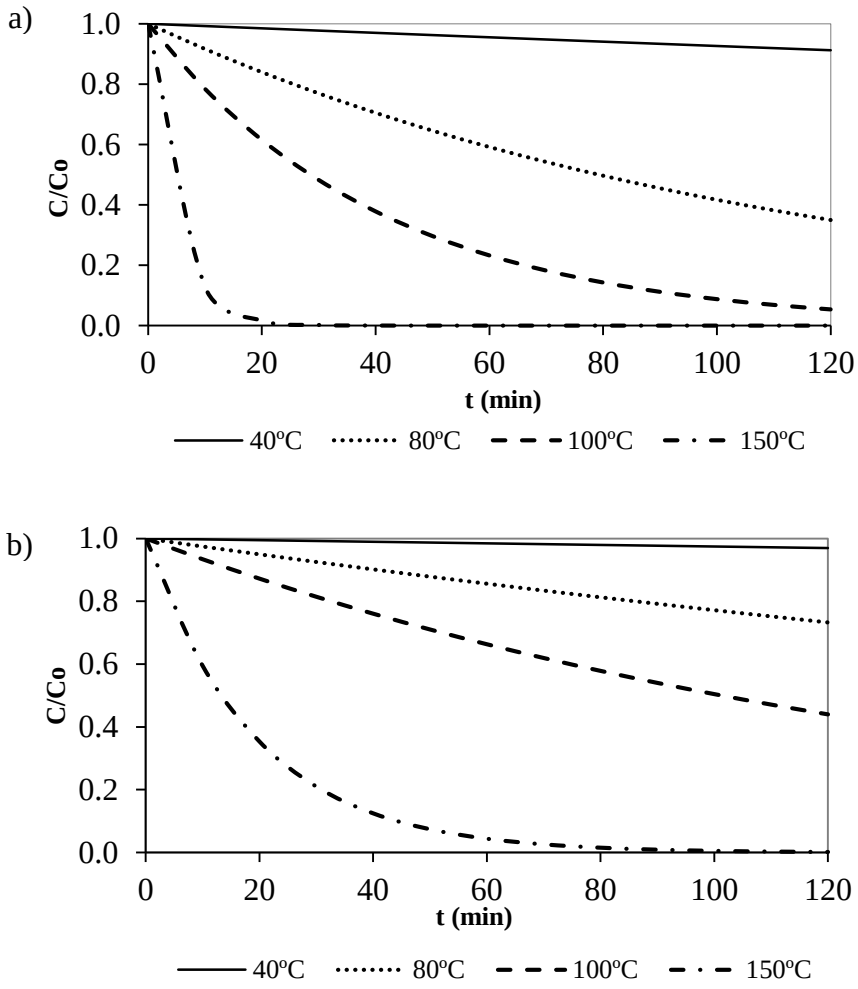


Figure 3 *Estimation of isothermal anthocyanin degradation kinetics at 40-80-100-150 °C for a) grape marc and b) filtered extract.*

Depending on the raw material and experimental conditions, such as pH, suspended solid concentration, and extraction solvents, 50% concentration reduction was found for blackberry juice after 4.7 h (*Wang and Xu, 2007*). In the case of dry bilberry extract this reduction was observed between 1 and 2 hours (*Yue and Xu, 2008*), 3.5 h in blood orange extract, and around 6 h for Roselle extract (*Cisse et al, 2009*). In the case of the grape marc thermal treatment (Figure 3 a), 3.5 minutes were enough to achieve 50% decrease at 150 °C, and 80 min at 80 °C.

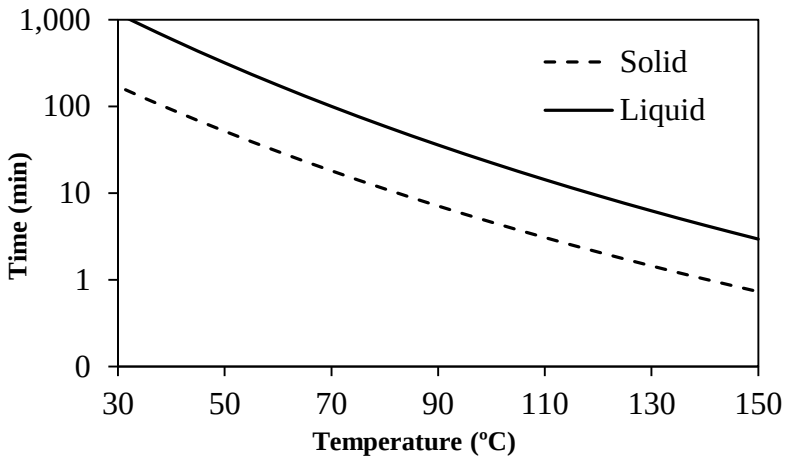


Figure 4 *Required combination of time and temperature for 10 % anthocyanin decrease in grape marc and filtered extract exposed to isothermal heating.*

For an efficient extraction process, more than 10% degradation of valuable compounds would not be convenient. Using the degradation kinetic parameters in isothermal simulation, 10% decrease in liquid and solid phase can be compared (Figure 4) at different temperatures. It is clearly seen that anthocyanin molecules suffer higher degradation in the presence of the solid phase, by one order of magnitude. These degradation rates should be taken into account in the development of new grape marc extraction processes in order to avoid major deterioration of anthocyanin in the material.

3.3. Total phenol content

In the case of thermal treatment on grape marc at 80 ºC (Figure 5a), significant decrease can be observed in TPC values. The slow heating up of the experimental setup could give rise to polyphenol oxidase enzyme activity in the material, lowering the phenol content in the first hour (*Kalt 2005*). Further degradation of TPC did not occur at 80 °C.

When heating up to 100 °C, TPC value was maintained for the first hour. However, a slight increment was achieved during the remaining time. Also increasing behaviour was detected at 150 °C for the first two hours, but a reduction took place later due to the thermal degradation of phenolic compounds. When grape seeds were heated before extraction, liberation of polyphenols was achieved at 100 °C and 150 °C after 60 and 40 minutes respectively (*Kim et al 2006*).

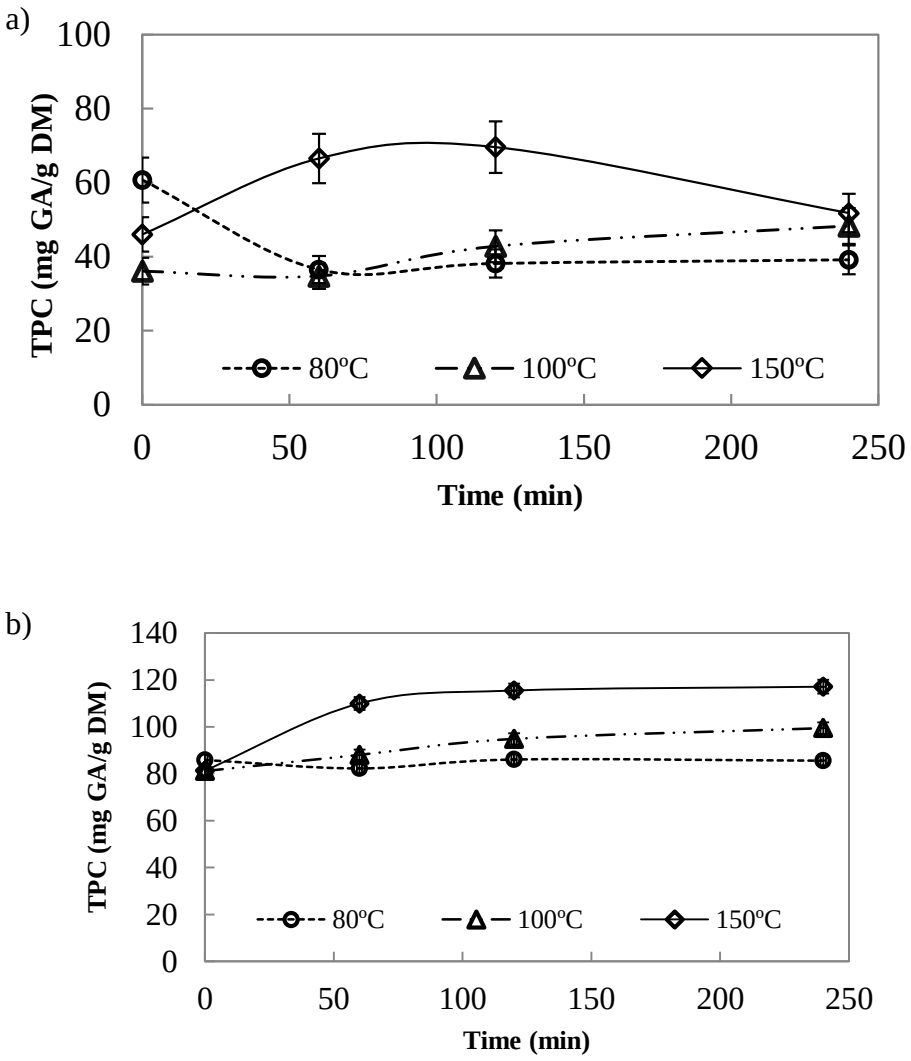


Figure 5 *Total phenol content of a) grape marc and b) filtered extract at 80-100-150 °C*

On the other hand, total extractable polyphenols of grape pomace peels decreased after drying at 100 °C and 140 °C, while no change was observed at 60 °C (*Larrauri 1997*).

The here studied raw material contains both, grape seeds and peels in the mixture, thus a combination can occur: on one hand initial thermal degradation of free polyphenols, and later degradation of bounded polyphenols as well. On the other hand, the release of bound polyphenol compounds and phenolic acid derivatives, because of partial lignin degradation in the seeds (*Maillard and Berset 1995*).

Filtered extracts did not behave the same way as solid grape marc. At 80 °C and 100 °C there was no TPC content change (Figure 5b). However, at 150 °C significant increment was achieved from 81.3±2.0 [*mg gallic acid/ g dry material*] of control value, to 117.1±2.9 after 4 hours. Enzyme activity did not affect the material, since the extraction previous to the thermal

treatment was performed at low temperature (40 °C) for 24 hours. Extracts from grape pomace after fermentation did not change in polyphenol concentration at 100 and 150 ºC, while at 200°C, 72% degradation was observed after 120 min (*Cruz et al, 2007*).

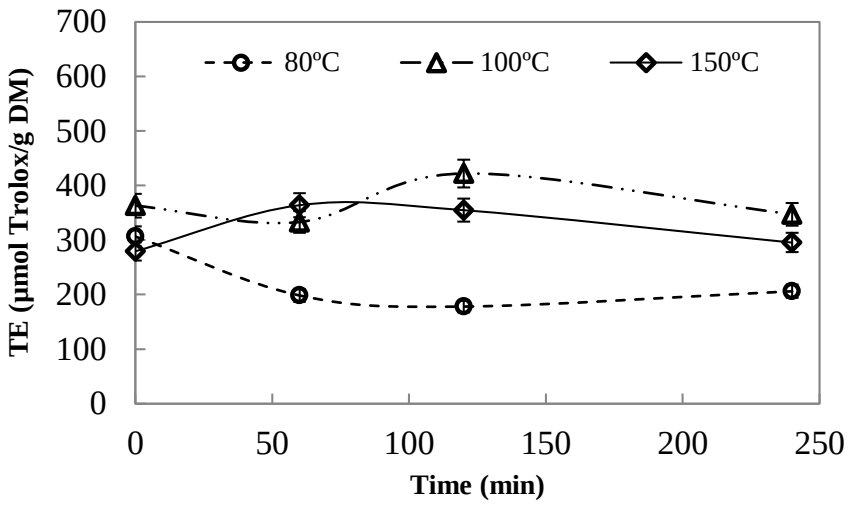
Phenolic compounds of grape marc contain a wide range of components, including anthocyanins, flavonols, falavan-3-ols and flavanonols. Mainly those are responsible for the nutritional quality, antioxidant activity, attributed to the positive health effect of this and similar natural materials (*Larrauri 1997*). Due to their diverse structure, different polyphenols are found in the distinct parts of the grape skin and seed. Some of them are free and can be found in vacuoles, while others are associated to cell wall compounds or to polysaccharide structures in the skin cells (*Pinelo et al, 2006*). The structural and bonding state of the different present polyphenols would explain the distinct overall behaviour of the total phenol concentration due to thermal treatments (*Kim et al, 2006*).

3.4. Antioxidant activity (ORAC)

Antioxidant power was analysed by the capacity to absorb oxygen radicals and compared to the power of known trolox concentration. Antioxidant activity of solid grape marc (Figure 6 a) showed similar behaviour to the one observed for TPC values. Treatment at 80 °C caused a slight decrease at the beginning of the process, but no further degradation happened.

The initial increment of ORAC values turned to a decomposition of active compounds in the second hour of the thermal treatment. Similar behaviour was found when drying methods were studied on grape skin with the ferric thiocyanate method (*Larrauri 1997*).

a)



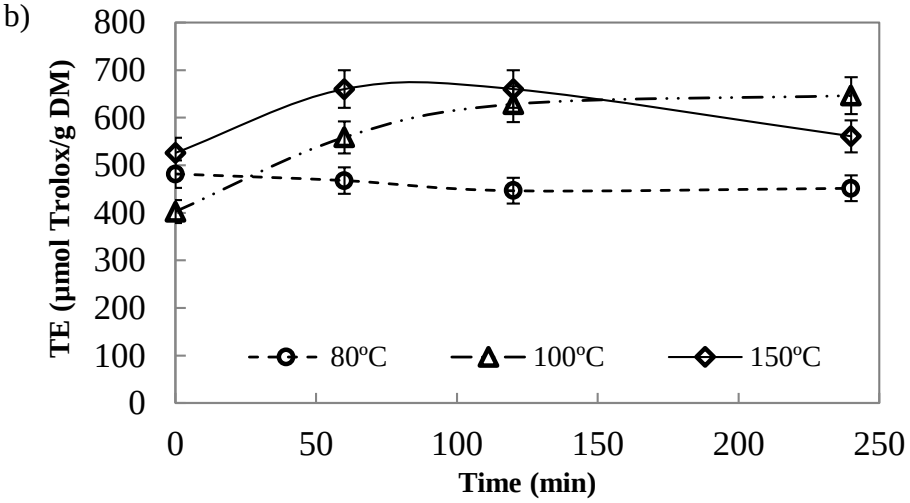


Figure 6 *Antioxidant activity of a) grape marc and b) filtered extract at 80-100-150 °C (ORAC values)*

Radical scavenging and reducing power of pre-heated grape seed was positively modified at 100 °C and 150 °C, while at 200 °C both values decreased (*Kim et al 2006*). In liquid extracts (Figure 6 b) the antioxidant activity did not change at 80 °C, while at 100 °C increment was achieved without final degradation. However degradation was once more observed at 150 °C. Radical scavenging power of wine making by-products and of bilberry extracts showed similar tendency after heat treatments (*Cruz et al, 2007, Yue and Xu, 2008*). Either the elevated reactivity of the degradation products or the activation of released bound phenolics could explain the results obtained here (*Maillard and Berset 1995, Guillot et al, 1996*). However, the exact composition and in vivo health benefits of the changed compounds need to be further studied.

4. CONCLUSIONS

This study explored the effects of heat treatments on a by-product of the wine making industry and its extract, in terms of anthocyanin degradation, total phenol content, and antioxidant activity. Anthocyanin degradation kinetics were studied under non-isothermal conditions, whereof isothermal treatment was estimated by first order kinetics and Arrhenius model. The presence of solvent showed to protect anthocyanin degradation from temperature. TPC and ORAC values also suggested that the semi-solid grape marc was more vulnerable to heat treatment than the isolated liquid extract, while an initial increase of concentration was observed over 100 °C. Process intensification methods for antioxidants extraction from grape marc must take into account the optimal matching between processing time, temperature and raw material conditioning in order to avoid degradation and enhance the antioxidant power of valuable compounds.

NOMENCLATURE

Abbreviations:

TPC	*total phenol content*	*mg gallic acid/ g dry material*
ORAC	*oxygen radical absorbance capacity*	*µmol trolox/ g dry material*
DM	*dry material*	

Parameters:

C	*anthocyanin concentration*	*mg gallic acid/ g dry material*
t	*time*	*min*
B	*degradation rate parameter*	min^{-1}
B_0	*pre-exponential constant of B*	-
$T_{initial}$	*temperature before heating treatment*	*K*
T_{final}	*final temperature after heating treatment*	*K*
k_T	*heating rate constant at corresponding* $T_{°C}$	min^{-1}
E_a	*energy of activation*	*J/mol*
R	*gas constant*	*8.314 J/molK*
χ^2	*sum of the squares of errors*	
n_j	*number of experimental data points of experiment j*	
N	*Total number of experimental data points with the same material (grape marc or liquid extract)*	
p	*number of correlation parameters*	
a_k	*parameter in standard error calculation (E_a or B_0)*	

ACKNOWLEDGMENTS

The authors thank the Spanish Ministry of Economy and Competitiveness for the Project CTQ2010-15475 and the project of ENE2012-33613.

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“... y en el mundo en conclusión todos sueñan lo que son, aunque ninguno lo entiende.”
/Calderón de la Barca/

Conclusions & future work

CONCLUSIONS

Through the former chapters, partial conclusions have been drawn corresponding to the ***successful process kinetics intensification*** on different raw materials using microwaves. In addition, other aspects were covered in connection with the interaction between the raw material and the dielectric field; with the application of ultrasound assisted extraction; and with the possible thermal deterioration of valuable compounds due to the pre-treatment processes.

In general, ***four main conclusions*** were drawn from the study of process kinetics enhancement by the application of microwaves to natural raw materials:

« The application of ***solvent free and solvent assisted*** microwave systems showed that the liberation of intracellular material might depend on the ***cell structures and the location therein of the compounds of interest***. In the case of wastewater sludge (*Chapter 1*) and microalgae samples (*Chapter 2*), the diminished water content (≈ 90%) was still high enough to enhance process kinetics after microwave irradiation. However, when grape marc was treated (*Chapter 4*) at high moisture content (≈ 85%) in the absence of solvent, positive results were not obtained and solvent addition was necessary during the microwave irradiation step in order to achieve a more effective extraction process, compared to the conventional method. The reason may be the difference between the simplified cellular structure of microorganisms and the higher-level cell organization of plant tissues.

« The use of ***microwave*** (*Chapter 4*) and ***ultrasound*** (*Chapter 5*) ***assisted extraction*** methods on polyphenol extraction from grape marc showed ***similar results*** in both cases. ***Temperature seemed to be the most important factor*** in both processes, while the effect of energy intensity (power) was not substantial. The measured thermal energy was found to be similar in ultrasound and microwave assisted extractions in order to obtain similar results. The non-thermal effect of the processes was discarded when compared to the influence of temperature. In the light of these results, advantages and disadvantages of process and equipment design must be explored in the comparison of the two novel process intensification technologies.

« The ***measure and evaluation of the absorbed energy*** amount in the different processes on a variety of raw materials was essential to compare results appropriately. In the case of

aqueous raw materials (wastewater sludge, microalgae), ***best results*** were obtained for an absorbed energy density around 0.4-0.5 kJ/ml, leading to a ***near boiling point*** condition. In the case of extraction processes from grape marc using an ethanol/water solvent mixture, a lower energy density was required to reach the highest temperature at atmospheric pressure (0.16-0.20 kJ/ml). Perhaps, either the boiling phenomena, or the fast heating up of the system, contributes to the success of the treatment. Comparing the energy efficiency of a domestic (40-50 %) and a laboratory microwave equipment (80-99%), the latter resulted to be more efficient. The difference might be due to the smaller cavity size and more focused irradiation, leading to better field homogeneity, less evaporation and negligible heat loss from the vessel surface. Here, a clear conclusion is the need of an experimental setup, where the absorbed energy density is measured, in order to get reliable results for up-scaling purposes.

« In anticipation of possible ***further scaling*** up on an industrial scale of the polyphenol extraction process from grape marc with the assistance of microwave irradiation, two other aspects were studied. On one hand, the ***interaction between the complex raw material and the electric field***, where it was found, that the ***importance of the moisture content*** present in the material overrides the influence of the temperature on the dielectric properties of the system (*Chapter 3*). On the other hand, a ***thermal degradation study on anthocyanins and polyphenols*** showed, that a ***short and high temperature pre-treatment*** does not lead to the deterioration of these valuable compounds in the liquid phase extract (*Chapter 6*).

FUTURE WORK

From the work presented here about the microwave effect on different processes, new research lines could complete the explored results:

« More detailed ***product and process specific analytical work*** might be performed in order to follow the changes in the components of interest for the process.

« As the structure of the raw material was found to be important, regarding to the success of the treatment in intracellular material liberation, a deeper study on the ***microwave effect on different cell structures*** would be useful, in order to clarify the cell rupture mechanism.

« Although a main objective of this work was to assess the use of solvent free microwave application to enhance process kinetics, in the case of the polyphenol extraction from grape marc the presence of the solvent was required during the irradiation in order to achieve that goal. Common diffusion phenomena and the elevated temperature cannot easily describe the extremely fast action of the extraction solvent. The exploration of ***the role of the solvent in microwave assisted extraction*** would give rise to another research line in conjunction with application studies on new products and processes.

« These results from microwave assisted extraction, dielectric properties and thermal degradation of grape marc polyphenols (*Chapters 3, 4 and 6*) will be used as starting point in one of the research lines of the ***Marie Curie Industry-Academia Partnerships and Pathways*** Project (IAPP, *Call: FP7-PEOPLE-2012-IAPP; Research on extraction and formulation intensification processes for natural actives of wine "****WineSense****"*). This research line of the project aims to develop an industrial scale microwave assisted extraction process of grape marc polyphenols.

Resumen Español

Extracción mejorada de sustancias naturales mediante energía de microondas

1. Introducción

Actualmente, en varios procesos industriales de extracción las nuevas tecnologías para reducir tiempo, espacio y energía, así como la cantidad de los disolventes utilizados, son bienvenidas como candidatas a la intensificación de la producción. Esto es especialmente importante en los casos complejos de materiales naturales donde la ruptura de las pareced celulares es la etapa limitante que conduce a cinéticas lentas y rendimientos bajos.

Entre varios tratamientos mecánicos, químicos, físicos o fisicoquímicos, está el uso de microondas en materias naturales. Debido a la irradiación electromagnética de la materia, el agua intracelular se calienta y se evapora rápidamente, y el gradiente de presión generado causa probablemente el daño de la pared celular. En consecuencia, los compuestos intracelulares de interés llegan a ser más asequibles con un pretratamiento eficaz, y se logra mejorar la cinética del posterior proceso de extracción convencional.

En esta Tesis Doctoral se han estudiado tres diferentes materias primas y dos procedimientos de pretratamiento para mejorar la cinética de extracción.

Primero se han utilizado microorganismos, en los lodos de aguas residuales, para mejorar la producción de biogás al liberar la materia intracelular. De esta forma se convierte en más digerible para las bacterias anaerobias en el proceso de la digestión. Debido a la gran variedad celular en las aguas residuales, resulta difícil estudiar la estructura de las células, aunque la cinética del proceso se puede mejorar en su conjunto, de forma análoga a un pretratamiento térmico.

En segundo estudio se ha realizado en microalgas unicelulares como mejores candidatos para este fin. También porque la extracción de lípidos para la producción de biodiesel y la extracción de pigmentos con alto valor añadido están en el centro de atención en el campo del desarrollo de los procesos sostenibles. El trabajo presentado usando diferentes microalgas y métodos de extracción demuestran que el pretratamiento por microondas es capaz de mejorar la cinética de extracción y además se alcanzaran mayores rendimientos.

Por último, la abundante viticultura de Castilla y León sugiere la investigación de los sub-productos de vinificación, como el hollejo de la uva, para extraer compuestos de alto valor (polifenoles) con elevada capacidad antioxidante. Comparado con las otras materias primas, el hollejo representa mayor nivel de organización celular donde el pretratamiento puede tener diferentes efectos sobre los diferentes tipos de paredes celulares. Se han estudiado los

pretratamientos por microondas con sistemas en presencia y ausencia del disolvente de extracción. El estudio comparativo muestra que la intensificación en la cinética de extracción solo se ha obtenido en tratamientos en presencia de disolvente. Como técnica competitiva alternativa a la de microondas, se ha estudiado el uso de ultrasonidos, cuyo resultado ha sido similar a la extracción asistida por microondas. Ambos pretratamientos disminuyeron los largos tiempos de extracción y consecuentemente la elevada cantidad de disolvente que se requiere en los procesos industriales. Además, se ha tenido en cuenta la posible degradación de los compuestos de alto valor.

Aparte del estudio de los procesos mencionados y del análisis del efecto de microondas sobre las diferentes materias primas se ha estudiado la energía absorbida por los materiales y disolventes durante el proceso. Una fracción de la energía electromagnética de microondas se absorbe por la materia y se disipa en forma de calor. Dicho calor puede describirse con un balance energético teniendo en cuenta el calor sensible asociado al incremento de la temperatura en el sistema, el calor latente absorbido por la evaporación del disolvente, y finalmente la perdida de calor hacia el ambiente. Por desgracia es difícil comparar los resultados publicados en la bibliografía entre sí sobre los temas mencionados por la falta de información referente a la energía absorbida. El conocimiento de la energía absorbida sería el método adecuado para obtener resultados comparables en diferentes condiciones experimentales, imprescindibles para escalar el proceso a nivel industrial.

Basándose en los resultados de esta disertación, como estudio adicional se podrá proceder al desarrollo de un proceso industrial de extracción asistida por microondas de polifenoles a partir del hollejo de uva. Además, el efecto de las microondas sobre las diferentes estructuras celulares y el rol del disolvente en la extracción asistida por microondas parecen ser temas de interés para comprender el papel de estos fenómenos, en un trabajo futuro.

2. Objetivos

El objetivo de esta Tesis Doctoral es el estudio del efecto de los pretratamientos por microondas en procesos con microorganismos (lodos de aguas residuales y microalgas) y con células vegetales, para facilitar la liberación de la materia intracelular y de este modo mejorar la cinética del proceso convencional de extracción que tiene lugar a continuación.

Con el fin de alcanzar el objetivo antedicho se han planteado los siguientes objetivos parciales:

- Estudiar los efectos de los pretratamientos de microondas en presencia (*Capítulo 4*) y ausencia de disolvente (*Capítulos 1, 2, 4*).
- Evaluar la aplicación de los ultrasonidos como una técnica novedosa y competitiva con la de microondas (*Capítulo 5*).
- Evaluar los aspectos que han de estudiarse previamente a nivel de laboratorio para poder realizar el escalado de los procesos:
  - Determinación de la energía absorbida a escala de laboratorio (*Capítulos 2, 3, 4, 5*).
  - Interacción entre las microondas y la materia prima para el posterior modelado del proceso (*Capítulo 3*).
  - Estudio de la degradación térmica de los compuestos de interés en diferentes ambientes experimentales (*Capítulo 6*).

3. Resultados y discusión

La tesis se divide en 6 capítulos para completar el estudio que se ha propuesto en el apartado de objetivos. Los siguientes apartados presentan un resumen de cada capítulo con los resultados más relevantes.

3.1. Influencia de la energía absorbida en los pre-tratamientos con microondas en la producción de biogás a partir de lodo segundario de aguas residuales

En este capítulo se ha estudiado el tratamiento con microondas para acelerar la etapa de hidrolisis en el proceso de digestión anaerobia de los fangos de aguas residuales municipales. Se ha investigado la influencia de la energía absorbida, la potencia y el efecto atérmico sobre la solubilización de la materia orgánica y sobre la producción de biogás. Además se ha propuesto un método novedoso para la determinación de la energía absorbida en sistemas experimentales de laboratorio (Figura 1), a fin de obtener resultados comparables entre diferentes equipos de microondas.

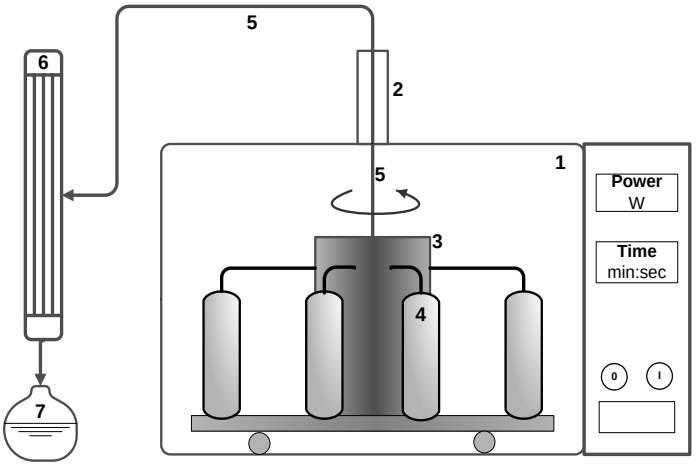

Figura 1. Horno de microondas para el tratamiento de lodos de aguas residuales: 1 – horno de microondas; 2 – tubo de salida, soldado a la cavidad de microondas; 3 – carrusel rotatorio con colección de vapor (r = 160 mm); 4 – tubos de policarbonato (85 ml, d= 38 mm); 5 – tubo de silicona para la salida de vapor; 6 – condensador; 7 – colector del condensado

La energía absorbida se calcula utilizando un balance energético. La mayor solubilización se ha obtenido utilizando 0,54 kJ/ml a 1000 W, donde la producción de biogás se ha aumentado un 7,1% comparado a una muestra sin tratamientos de microondas. Al aumentar la energía

aplicada (0,83 kJ/ml) la producción de biogás seguía aumentando (15,4%), aunque el valor de solubilización disminuyera (Figura 2).

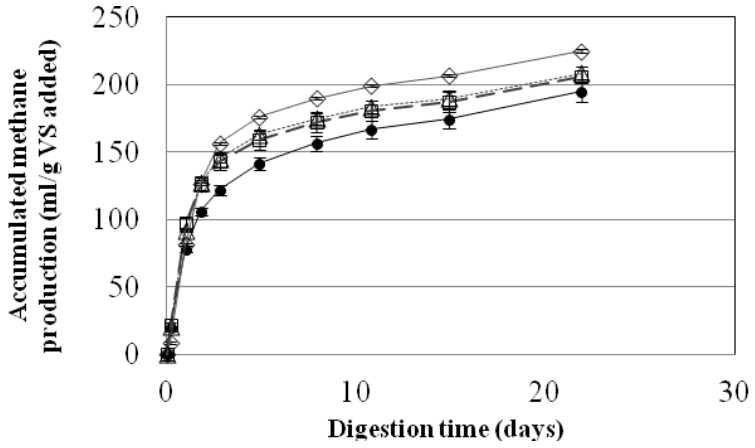

Figura 2. Producción acumulado de CH_4 (ml/g VS añadido) para muestras de control y tratamientos de 0.40, 0.54 and 0.83 kJ/ml energía absorbida a una potencia constante (1000 W). (-●-): Control, muestra sin tratamiento; (- -□- -): 0.40 kJ/ml; (••Δ••): 0.54 kJ/ml; (--◊--): 0.83 kJ/ml

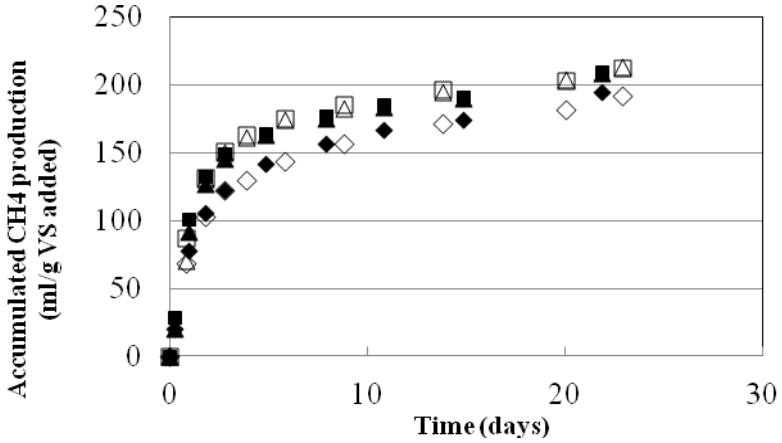

Figura 3. Producción acumulada de CH_4 (ml/g VS añadido) para la comparación de calentamiento convencional y por microondas. (♦): Control 1; (▲): MW 1; (■): CH 1; (◊): Control 2; (Δ): MW 2; (□): CH 2.

No se ha observado influencia de la potencia al comparar experimentos a 1000, 600 y 440 W con 0,54 kJ/ml. El calentamiento por microondas se ha comparado con el calentamiento convencional en dos sistemas diferentes (Figura 3), obteniéndose resultados similares en todos los casos.

3.2. Intensificación de la cinética de extracción de lípidos y pigmentos a partir de microalgas aplicando microondas

Los compuestos biológicamente activos y la producción de biogás a partir de microalgas son de gran interés para la industria, dando lugar a un amplio campo de investigación tanto en los procesos "upstream" como en los de "downstream". En este capítulo se ha propuesto la aplicación de un pretratamiento de microondas para la mejora de la cinética de extracción de los lípidos y colorantes a partir de las algas *Nannochloropsis gaditana* y *Scenedesmus alemriensis,* respectivamente.

Después de los pretratamientos de microondas a 65-80-88°C de *Nannochloropsis*, el rendimiento de la extracción se ha duplicado utilizando el Soxhlet con hexano como método de extracción (Figura 4). El daño de la superficie de las células se ha puesto en evidencia mediante microscopia electrónica de barrido (Figura 5).

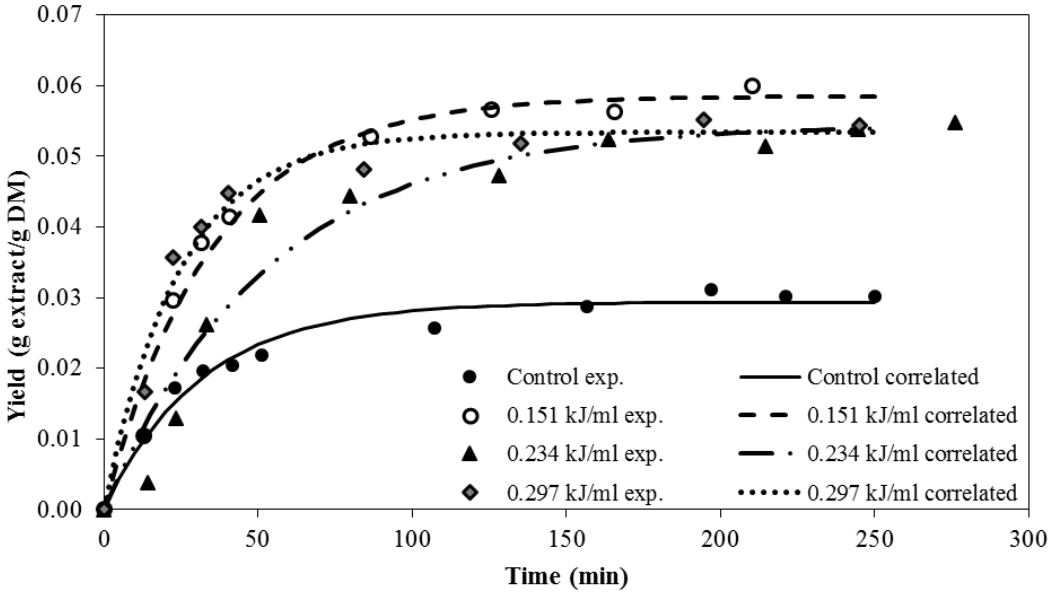

Figura 4. Datos experimentales y correlacionados con una cinética de extracción de primer orden de la obtención de lípidos de Nannochloropsis gaditana (espectrofotometría).

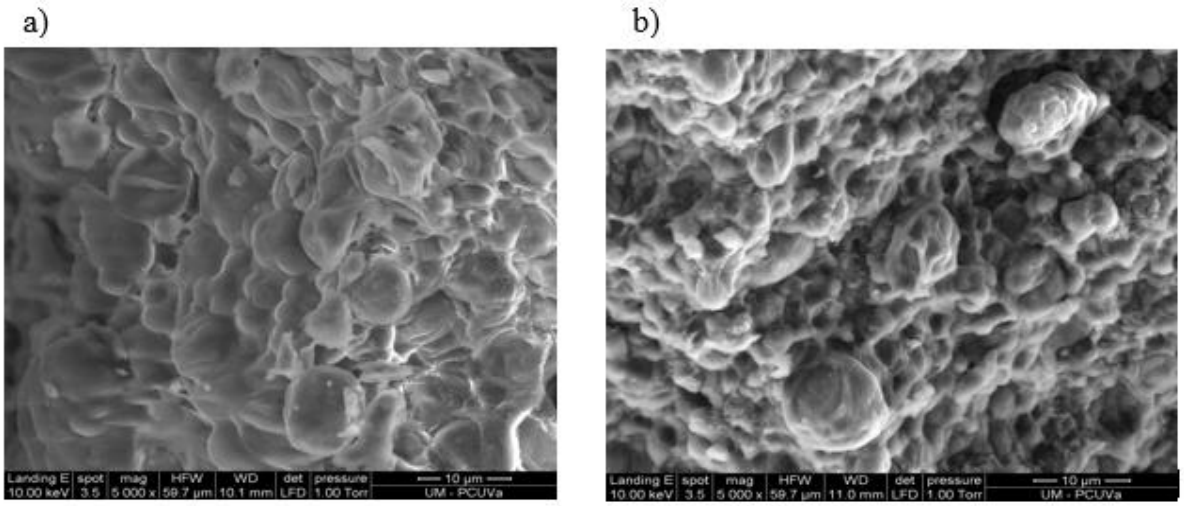

Figura 5. Imagen de microscopia electrónica de barrido de Nannochloropsis gaditana: a) antes y b) después del tratamiento con microondas

En el caso de las algas *Scenedesmus* los pretratamientos a 47-68-86°C han producido un incremento de 33-38-161% en la absorbancia a 440 nm de longitud de onda (Figura 6). En este caso se ha utilizado un método de extracción con agitación, empleando algas húmedas y hexano como disolvente, durante un periodo de 24 horas.

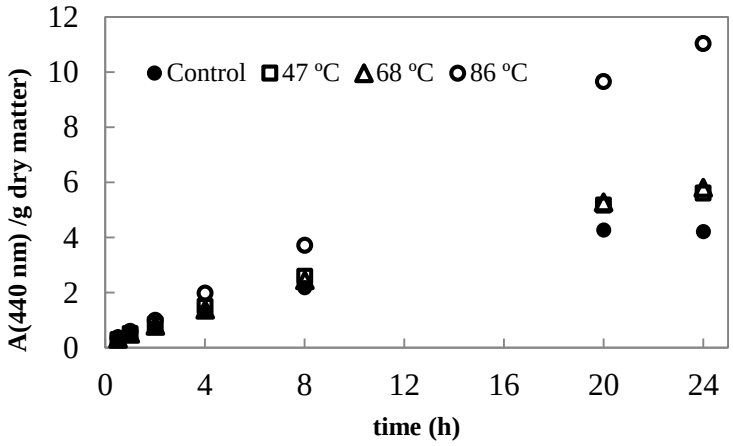

Figura 6. Resultados experimentales de la cinética de extracción de pigmentos a partir de Scenedesmus almeriensis a diferentes temperaturas, comparados con una muestra sin pretratamiento (Control).

El análisis de los ácidos grasos poliinsaturados ha demostrado que no se ha producido degradación térmica en dichos compuestos después del tratamiento de microondas a 86°C.

Independiente de las diversas condiciones experimentales, el tratamiento de microondas ha demostrado ser exitoso en la intensificación de los procesos "downstream".

3.3. Propiedades dieléctricas del orujo de uvas: efecto de la temperatura, la humedad y el método de preparación de la muestra

La extracción asistida por microondas puede llevar a mejores rendimientos y cinéticas más rápidas en la extracción de antioxidantes a partir del hollejo de la uva. Para alcanzar un proceso eficaz de microondas, se deben medir las propiedades dieléctricas de la materia en diferentes condiciones a fin de poder implementar esta información en el diseño del horno microondas. Este capítulo se ha centrado en la medida de las propiedades dieléctricas del hollejo de la uva (mezcla de pepitas y piel de uva) utilizando un aparato de cavidad resonante. Como la preparación de la muestra muchas veces incluye una etapa de molida para obtener muestras homogéneas, también se ha estudiado el efecto de esta etapa.

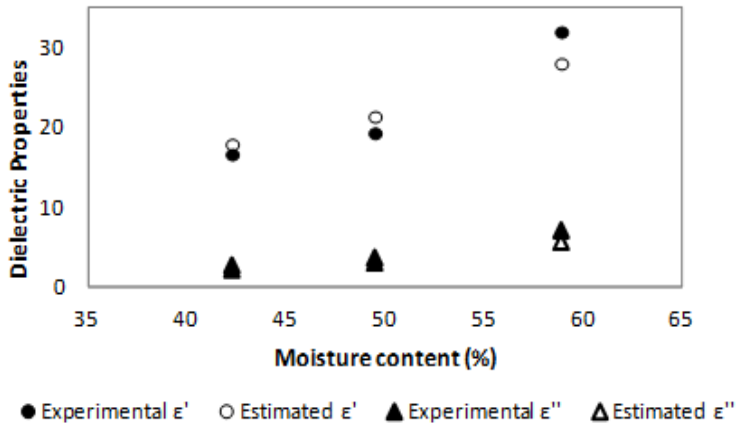

Figura 7. Comparación de las propiedades dieléctricas (T=28°) de hollejo de uva molido a porosidad cero, extrapolado de los valores experimentales ('Experimental'), y de la estimación de la ecuación CRIM a partir de los constituyentes de pepita y piel ('Estimated')

Los resultados experimentales se han comparado utilizando ecuaciones de mezcla. En el rango de porosidad entre 0,4 y 0,6 la estimación de la ecuación compleja del índice de refracción fue la más adecuada. También se ha obtenido una buena correlación con la misma ecuación en el caso de la comparación entre la mezcla física de las pepitas y piel de uva y la mezcla estimada basada en las medidas separadas de las diferentes partes del hollejo (Figura 7). La comparación demuestra que los valores obtenidos están cerca de los valores reales del hollejo de la uva, a pesar de la complejidad del método de medida.

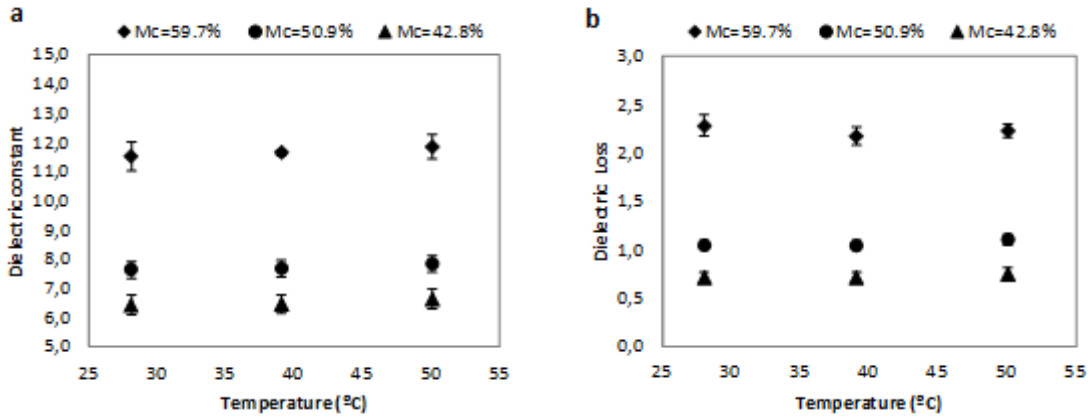

Figura 8.Constante dieléctrica (a) y pérdida dieléctrica (b) del hollejo de uva molido (mezcla de pepita y piel)

Se han estudiado tres diferentes niveles de humedad para las muestras de pepitas, piel y su mezcla a 28, 39 y a 50°C. El incremento de la humedad ha resultado en un aumento del valor en las propiedades dieléctricas, sin embargo no se ha observado un efecto definitivo de la temperatura (Figura 8).

3.4. Efecto de la energía absorbida de microondas en las cinéticas de extracción de los compuestos fenólicos a partir del orujo de uva

El hollejo de la uva, subproducto de la vinificación, parece ser buen candidato para la obtención de compuestos con actividad biológica, de gran interés para la industria alimentaria, farmacéutica y cosmética. El proceso convencional requiere largos tiempos de extracción y cantidades excesivas de disolvente, lo cual provoca la necesidad de tecnologías novedosas, como la de microondas, para mejorar la cinética de la extracción. En este capítulo, el estudio de la cinética de extracción asistida por microondas se ha descrito con una cinética de primer orden, para diferentes niveles de potencia (80-150-300 W) y energía absorbida (≈70 – 120 - 200 J/ml) (Tabla 1). Como estudio preliminar también se ha propuesto la aplicación de microondas como pretratamiento sin disolvente, sin embargo este proceso no ha mejorado el subsiguiente proceso convencional.

Power	*E density*	*Yo* $_{TPC}$	*σ (Yo)*	*Yf* $_{TPC}$	*σ (Yf)*	*β* $_{TPC}$	*σ (β)*	R^2	*Yo* $_{AC}$	*σ (Yo)*	*Yf* $_{AC}$	*σ (Yf)*	*β* $_{AC}$	*σ (β)*	R^2
W	*kJ/ml*	*mg GA/g DM*		*mg GA/g DM*		*min* $^{-1}$		*%*	*mg CG/g DM*		*mg CG/g DM*		*min* $^{-1}$		
80	0.201	1.75	0.38	7.75	0.37	0.181	0.024	96.3	9.73E-04	2.0E-04	1.85E-03	1.9E-04	0.418	0.076	91.4
80	0.147	2.89	0.32	5.32	0.31	0.186	0.032	97.8	1.57E-03	2.7E-04	1.66E-03	2.5E-04	0.216	0.088	87.5
80	0.074	2.54	0.29	3.10	0.28	0.233	0.053	88.7	1.46E-03	8.5E-05	1.01E-03	8.6E-05	0.184	0.046	89.1
150	0.194	1.84	0.59	7.04	0.56	0.220	0.048	91.2	1.06E-03	1.9E-04	2.05E-03	1.8E-04	0.357	0.061	92.6
150	0.125	1.28	0.41	5.84	0.38	0.341	0.043	98.1	7.63E-04	1.9E-04	2.16E-03	1.8E-04	0.402	0.059	97.3
150	0.070	2.89	0.26	3.72	0.25	0.256	0.044	97.5	1.63E-03	1.6E-04	1.43E-03	1.5E-04	0.276	0.069	94.8
300	0.186	0.68	0.44	6.50	0.41	0.310	0.042	98.3	3.98E-04	1.3E-04	1.98E-03	1.2E-04	0.560	0.051	99.2
300	0.118	1.42	0.61	5.26	0.57	0.370	0.073	96.2	9.23E-04	1.9E-04	1.71E-03	1.8E-04	0.459	0.075	97.3
300	0.067	2.42	0.26	3.57	0.24	0.272	0.043	97.9	1.16E-03	6.7E-05	1.21E-03	6.2E-05	0.337	0.035	99.1
Control	-	1.97	0.77	7.96	0.36	0.057	0.010	94.5	9.23E-04	1.2E-03	2.27E-03	1.0E-04	0.090	0.018	89.6
Control	-	1.97	0.77	6.72	0.44	0.113	0.031	75.7	9.23E-04	1.2E-03	2.01E-03	5.0E-05	0.164	0.023	88.7

Tabla 1. Parámetros de la cinética de primer orden para extracción asistida por microondas y experimentos de control.

La extracción completa de los fenoles totales se ha observado a 200 J/ml de energía absorbida, aunque las antocianinas se han extraído rápidamente a todas las energías aplicadas. Comparado con el proceso convencional, la cinética de extracción de los fenoles totales y de las antocianinas fue 4 y 4,5 veces más rápida, respectivamente (Figura 9). El coste energético requerido para dicho tratamiento de microondas fue estimado en 0,03 €/kg hollejo húmedo.

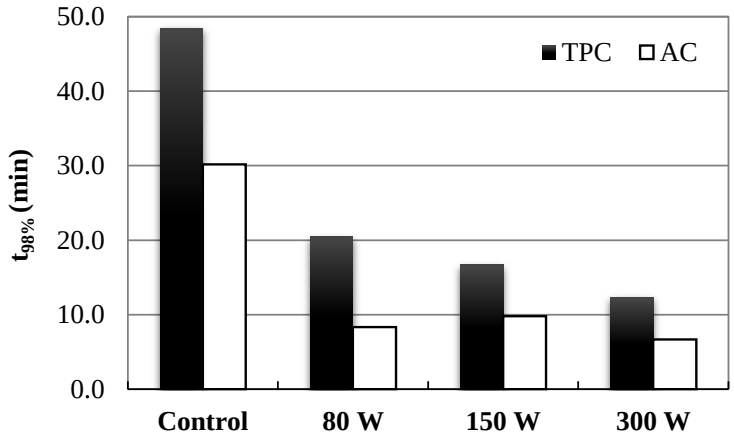

Figura 9. Tiempo requerido para alcanzar 98% del rendimiento máximo (TPC: fenoles totales, AC: Antocianinas) en el control y en los experimentos de extracción asistida por microondas ($E_{density}$≈200 J/ml) a diferentes potencias

3.5. Extracción mejorada de polifenoles a partir del orujo de uvas después del pre-tratamiento con ultrasonidos

La extracción asistida por ultrasonidos es conocida por su capacidad para mejorar la transferencia de materia lenta, inherente a los procesos convencionales, como la extracción de antioxidantes a partir del hollejo de la uva. En este capítulo se aplicaron los ultrasonidos como tratamiento previo a la extracción convencional de los polifenoles. De esta forma se puede mejorar la cinética de la extracción utilizando menos energía que en otros procesos de extracción. Eso facilitaría su posible implementación a escala industrial.

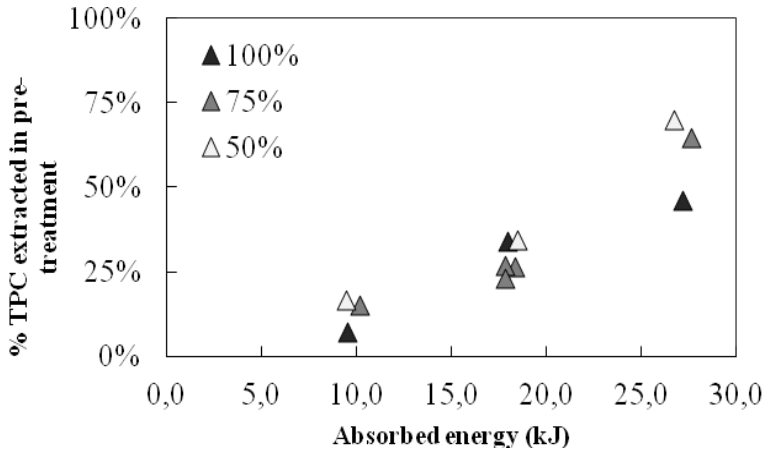

Figura 10. Rendimiento de fenoles totales representado como porcentaje del rendimiento total a diferentes energías absorbidas y niveles de intensidad

Se han estudiado los efectos de la energía absorbida y de la intensidad de esta energía. El incremento de la temperatura (40-55-70°C) ha causado una mejora significativa, sin embargo la intensidad de la energía (100-75-50%) ha demostrado poco efecto sobre el rendimiento final o sobre la velocidad inicial de extracción. Hasta un 70% del rendimiento final se ha obtenido solamente durante el pretratamiento (Figura 10).

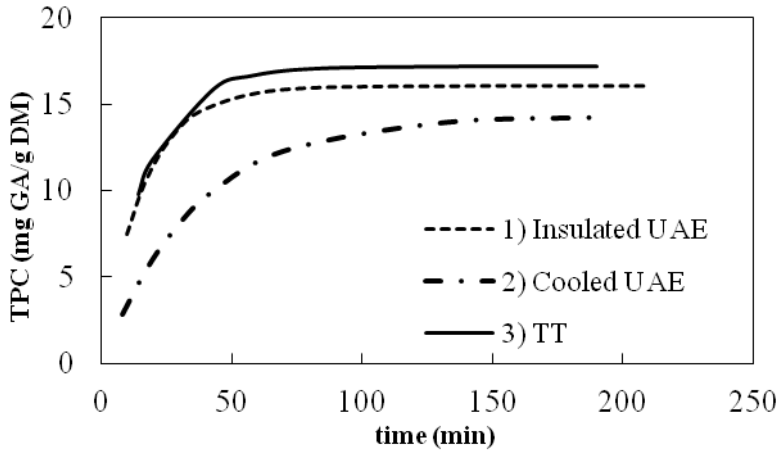

Figura 11. Cinética de extracción después del pretratamiento 1) UAE aislado a 70ºC, 2) UAE refrigerado utilizando la misma energía, y 3) pretratamiento térmico (TT) a 70ºC.

Tras la comparación con un pretratamiento por calentamiento convencional no se pudo demostrar la evidencia de un efecto específico de los ultrasonidos diferente al efecto térmico (Figura 11).

3.6. Degradación térmica de polifenoles del orujo de uvas

Los compuestos biológicamente activos de los subproductos de la vinificación son de gran interés en varios campos industriales. No obstante, los procesos convencionales de extracción necesitan tiempos largos y uso excesivo de disolventes, dando lugar al desarrollo de la intensificación de los procesos convencionales. Estas técnicas a menudo conllevan la aplicación de temperaturas altas que pueden empeorar la calidad del extracto.

En este capítulo se ha estudiado un tratamiento térmico (80-100-150°C, hasta 4 horas de duración) sobre el hollejo semisólido de la uva y sobre el extracto líquido de la misma. Se ha descrito la disminución del contenido en antocianinas en condiciones no-isotérmicas con un modelo de cinética de primer orden y con la ecuación de Arrhenius.

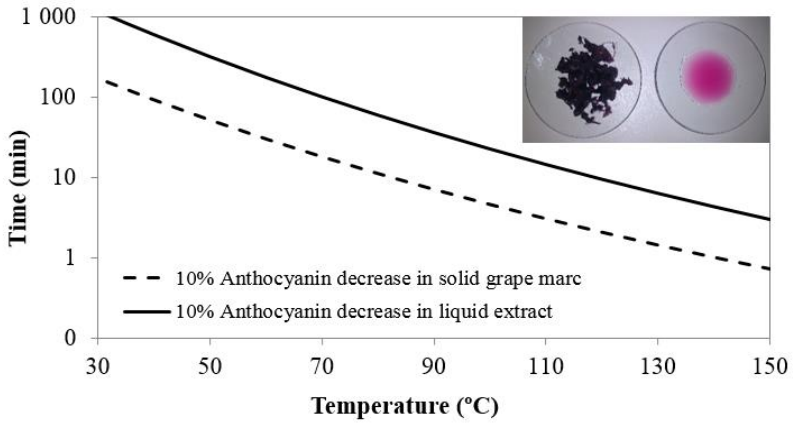

Figura 12 Combinación de tiempo y temperatura para un 10% de degradación de antocianinas en el hollejo semisólido y en el extracto líquido.

Los parámetros estimados para condiciones isotérmicas han demostrado que la materia prima en estado semisólido es más sensible al calor, mientras el extracto líquido es capaz de soportar tratamientos prolongados un orden de magnitud superior (Figura 12). A pesar del incremento inicial en el contenido de fenoles totales y en la actividad antioxidante, los análisis han confirmado la vulnerabilidad del hollejo semisólido. Una combinación óptima de temperatura, tiempo de tratamiento y entorno de la materia prima es necesaria para establecer la intensificación de los procesos.

4. Conclusiones y trabajo futuro

Conclusiones

Durante los capítulos anteriores se ha llegado a conclusiones parciales correspondientes a la intensificación de la cinética de los procesos por microondas a partir de diferentes materias primas. Además se han estudiado otros aspectos como la interacción entre la materia prima y el campo dieléctrico; la aplicación de la extracción asistida por ultrasonidos; y el posible deterioro térmico de los compuestos de alto valor debido a los pretratamientos.

En general se concluyen cuatro aspectos principales del estudio de la mejora de la cinética de procesos mediante la aplicación de microondas sobre materias primas naturales.

» La aplicación de microondas a sistemas con y sin disolvente ha demostrado que la liberación de la materia intracelular podría ser dependiente de la estructura celular y de la ubicación de los compuestos de interés. En el caso de los lodos de aguas residuales (*Capítulo 1*) y de las microalgas (*Capítulo 2*) el contenido disminuido en agua (de 99,9 a 90%) era suficiente para la intensificación de la cinética de los procesos después de la irradiación por microondas. Sin embargo, cuando se ha tratado el hollejo de la uva de alta humedad (≈ 85%), la ausencia de disolvente no ha llevado a resultados positivos y ha sido necesario añadir disolvente durante la etapa de irradiación por microondas para alcanzar una extracción más eficaz que el método convencional. La razón puede estar en la diferencia entre la estructura celular sencilla de los microrganismos y la organización de alto nivel de las células vegetales.

» En el uso de microondas (*Capítulo 4*) y ultrasonidos (*Capítulo 5*) en la extracción de polifenoles a partir de hollejo de uva se han obtenido resultados similares en ambos casos. El efecto de la temperatura parecía ser el más importante en ambos procesos, mientras que el efecto de la intensidad de energía (potencia) es menos relevante. La energía térmica medida ha resultado ser similar en ambos procesos, microondas y ultrasonidos, para obtener resultados similares. Se ha descartado el efecto atérmico tras la comparación con la influencia de la temperatura. A la vista de estos resultados, para la comparación de las dos tecnologías novedosas para la intensificación del proceso se deberían evaluar las ventajas y desventajas en el diseño de los procesos y los equipos correspondientes en cada caso.

» La medida y evaluación de la energía absorbida en los diferentes procesos resulta esencial para una comparación adecuada. En el caso de las muestras acuosas (lodos de aguas residuales y microalgas) los mejores resultados se han obtenido con una densidad de energía absorbida alrededor de 0,4 – 0,5 kJ/ml, que ha llevado las muestras cerca de su punto de ebullición. En el caso de los procesos con el hollejo de uva, cuando el disolvente era una mezcla de etanol y agua, una menor densidad de energía absorbida resultó necesaria para alcanzar la mayor temperatura a presión atmosférica (0,16-0,20 kJ/ml). Probablemente el hecho de la ebullición o el calentamiento rápido contribuyen a la eficacia del tratamiento. Comparando las eficacias energéticas entre el equipo doméstico (40-50%) y el equipo del laboratorio (80-99%), este último ha sido claramente más eficaz. La diferencia puede ser debida al menor tamaño de la cavidad y a una irradiación más concentrada, lo cual permite alcanzar un campo más homogéneo y se observa menos evaporación del disolvente y menos pérdidas de calor en la superficie. En este asunto la conclusión más importante es la necesidad de un montaje experimental donde se pueda medir la densidad de la energía absorbida para obtener datos fiables con la finalidad de proceder al diseño a mayor escala.

» Para un posible diseño industrial del proceso de extracción asistida por microondas de polifenoles a partir del hollejo de la uva, se han estudiado otros dos aspectos. Por un lado la interacción entre la materia prima compleja y el campo dieléctrico, donde se ha observado que el contenido de humedad presente en la materia tiene mayor influencia que la temperatura de la muestra, en lo que se refiere a la medida de las propiedades dieléctricas (*Capítulo 3*). Por otro lado, el estudio de la degradación térmica de las antocianinas y otros polifenoles ha demostrado que un pretratamiento corto a temperaturas altas no causaría el deterioro de estos compuestos de alto valor en una fase liquida del extracto (*Capítulo 6*).

Trabajo futuro

Basado en el trabajo presentado sobre el efecto de microondas en diferentes procesos, nuevas líneas de investigación podrían completar los resultados obtenidos:

» Se podría proceder a un estudio de análisis más detallado y especifico de las materias primas para seguir los cambios de los compuestos de interés para el proceso.

» Puesto que se ha comprobado la importancia de la estructura de las células en el éxito del pretratamiento para la liberación de la materia intracelular, podría ser útil un estudio que se centrara en el efecto de las microondas sobre las diferentes estructuras celulares, para aclarar el mecanismo de ruptura de la pared celular.

» Aunque el objetivo principal de este trabajo ha sido la aplicación de microondas sin disolvente para mejorar la cinética del proceso, en el caso de la extracción de polifenoles a partir del hollejo de la uva la adición del disolvente fue necesaria para alcanzar esta meta. Con los fenómenos comunes de difusión y la operación a temperatura elevada no se puede describir fácilmente la acción tan rápida del disolvente en la extracción. Por este motivo, la exploración del rol del disolvente en la extracción asistida por microondas podría dar lugar a otra línea de investigación, junto a estudios aplicados sobre nuevos productos y procesos.

» Los resultados de la extracción asistida por microondas, de la medida de las propiedades dieléctricas y de la degradación térmica de los polifenoles del hollejo de la uva (Capítulos 3, 4, y 6) se van a utilizar como punto de partida en uno de las líneas de la acción ***Marie Curie Industry-Academia Partnerships and Pathways*** Project (IAPP, *Call: FP7-PEOPLE-2012-IAPP; Research on extraction and formulation intensification processes for natural actives of wine "**WineSense**"*). Esta línea del proyecto tiene como objetivo el desarrollo de un proceso de extracción asistida por microondas de los polifenoles del hollejo de la uva a escala industrial.

“When we dream alone, it is only a dream, but when many dream together it is the beginning of a new reality.” /Hundertwasser/

Acknowledgements

Rafa, María José, en primer lugar quiero daros las gracias por dar ejemplo tanto profesional como personalmente, y por la dirección de mi trabajo a lo largo de estos años. Dirección que abarcaba vuestro valioso tiempo y paciencia en la enseñanza, la motivación y la confianza que me han acompañado durante la tesis.

I am very grateful to Professor Heike P. Schuchmann for giving me the possibility of doing a research collaboration in her group and to Dr. Volker Gaukel and Dipl.-Ing. Stefan Kraus for the guidance and advice during my stay.

Me gustaría agradecer al programa de becas FPI UVa (Formación de personal investigador de la Universidad de Valladolid) por la financiación recibida durante la tesis y las estancias realizadas en el extranjero.

Gran parte de la experimentación no se hubiera podido llevar a cabo sin la ayuda de Javier Pereda y Miguel Ángel Álvarez con las algas, y de Marie Sophie Haverkamp, Ruth Solá Macías, Dániel Varga, Sara Pérez Nieto y Jorge Fernández todos ellos trabajando con hollejo de uvas. Muchas gracias a todos vosotros por el imprescindible trabajo.

Gracias a Araceli, Dani, Álvaro y Concha nunca he quedado sin consejos en los análisis de las muestras, problemas informáticos, montajes experimentales y luchas interminables con los papeleos.

Agradezco a los compañeros del grupo HPP y del Departamento de IQTMA todas las experiencias compartidas tanto en horario de trabajo como fuera de ellos.

Alex y familia, Danilo, João, Oscar, Pana, Raquel y Salima, muchas gracias como compañeros y amigos por compartir ideas, culturas (en todos sus sentidos), experiencias, y por vuestra presencia en los momentos claves.

Hálás köszönettel tartozom Dr. Simándi Béla Tanár Úrnak, amiért szárnyai alatt elkezdhettem, és annyira megszerettem a kutatást, továbbá, amiért általa kapcsolatba kerültem a *High Pressure Process* csoporttal Valladolidban.

Kedves kis családom és legközelebbi barátaim: köszönöm, és örök hálám amiért mindig mellettem álltok, és bátorítotok, mintha a fizikai távolság nem is létezne.

“If we do not honor our past, we lose our future. If we destroy our roots, we cannot grow.” /Hundertwasser/

About the author

Katalin Sólyom

About the author

Katalin Sólyom (1985, Budapest, Hungary), due to her interest in natural sciences since the high school, continued her studies at the Budapest University of Technology and Economics as a Bioengineer (2003-2009). During the third year of the university, she joined to the group of Professor Béla Simándi as a student assistant, where she first got in touch with research, and extraction techniques of natural products. As a practical training in 2006, she spent 4 months at the Wageningen University and Research Centre (The Netherlands) working on extraction processes also from natural sources. On the advice of Professor Simándi, she gladly applied to the Erasmus Programme at the University of Valladolid (Spain) for the preparation of her master thesis in the High Pressure Process Group of Professor María José Cocero under the supervision of Professor Rafael B. Mato and Alexander Navarrte on the topic of solvent free microwave extraction.

Thanks to the positive interplay of the conditions, after graduating as a Bioengineer in Budapest, she started her PhD studies (2009) under the direction of Professor Rafael B. Mato and Professor María José Cocero. Along the last years, thanks to the PhD scholarship of the University of Valladolid, she took part in two research stays in Karlsruhe (KIT; LVT), Germany.

The keyword of her short research experience so far is *natural products*, which is still the focus of interest for Katalin in the field of process engineering.

In personal life she adores sports, especially capoeira and outdoor activities, music, confectionery and gastronomy.

Selected works:

» Sólyom, K., Mato, R. B., Pérez Elvira, S. I., Cocero, M. J. (2011) *The influence of the energy absorbed from microwave pre-treatment on biogas production from secondary wastewater sludge*. Bioresource Technology.102. 10849 - 10854.

» Sólyom, K., Pereda, J., Mato, R. B., Cocero, M. J. (2011) *Microwave enhanced lipid extraction of microalgae* (poster) 13th International Conference on Microwave and Radio Frequency Heating, Toulouse, France.

» Sólyom, K., Kraus, S., Mato, R. B., Gaukel, V., Schuchmann, H. P., Cocero, M. J. (2013) *Dielectric properties of grape marc: effect of temperature, moisture content and sample preparation method*. Journal of Food Engineering. 119. 33-39.

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