PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

ESCUELA DE INGENIERIA

PRESSURIZED HOT WATER EXTRACTION OF POLYPHENOLS FROM AGROINDUSTRIAL BY-PRODUCTS: BIOACTIVITY ASSESSMENT

JOSÉ RODRIGO VERGARA SALINAS

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences

Advisor:

JOSÉ RICARDO PÉREZ CORREA

Santiago de Chile, Marzo, 2014

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Dedicado a mi hija Rafaella y
a la memoria de mi abuelo Jorge.

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EXTRACCIÓN CON AGUA CALIENTE PRESURIZADA DE POLIFENOLES DE SUBPRODUCTOS AGROINDUSTRIALES: EVALUACIÓN DE LA BIOACTIVIDAD

Tesis enviada a la Dirección de Investigación y Postgrado en cumplimiento parcial de los requisitos para el grado de Doctor en Ciencias de la Ingeniería.

José Rodrigo Vergara Salinas

RESUMEN

Suplementar la dieta humana con alimentos ricos en antioxidantes naturales previene el desarrollo de varias enfermedades relacionadas con la oxidación. El más abundante de los antioxidantes presentes en las plantas comestibles y no comestibles, así como en los residuos generados a partir de su procesamiento, son los polifenoles. Los compuestos fenólicos presentan actividades biológicas que los hacen atractivos para aplicaciones medicinales y nutracéuticas: previenen el daño celular por oxidación, actúan como antimutágenos, anticancerígenos y agentes antimicrobianos, y son potentes antioxidantes. Desde el punto de vista económico la recuperación de estos compuestos bioactivos a partir de subproductos agroindustriales mediante procesos limpios, inocuos y de bajo costo, es fundamental para el desarrollo y masificación de productos nutracéuticos. La extracción con agua a alta temperatura y presión ha surgido como una alternativa muy atractiva al uso de solventes orgánicos. Varias propiedades fisicoquímicas del agua, tales como la polaridad, la tensión superficial y la viscosidad pueden ser manipuladas a través del cambio en la temperatura (manteniendo el agua en estado líquido) con el fin de mejorar la solubilidad y difusión de compuestos de interés.

El proceso de extracción con agua caliente presurizada (EACP) brinda altos rendimientos y posibilita la obtención selectiva de compuestos en cortos tiempos de proceso. Sin embargo, las altas temperaturas también causan la degradación de compuestos termolábiles y la formación de nuevos compuestos antioxidantes, que en algunos casos pueden ser perjudiciales para la salud. En esta tesis se determinaron los efectos de los principales parámetros en el proceso de EACP (temperatura y tiempo) en el rendimiento de extracción de polifenoles y de antioxidantes totales de dos subproductos agro-industriales (tomillo desodorizado y orujo de uva). Luego, dos extractos de orujo de uva obtenidos a diferentes temperaturas (100 y 200 °C) fueron caracterizados en términos de actividad antioxidante biológica y química, así como su contenido de antioxidantes fenólicos y no fenólicos.

En general, los flavonoides simples (flavonas, flavonoles, flavanonas y antocianinas) y ácidos fenólicos alcanzan un rendimiento de extracción máximo a los 100 °C. El uso de temperaturas más altas (por encima de 100 °C) y largos tiempos de extracción producen la degradación de estos compuestos. Mientras que los flavonoides poliméricos (taninos condensados) presentan un rendimiento de extracción máximo a los 150 °C evidenciando una alta estabilidad térmica (posiblemente atribuible a su estructura polimérica). Las altas temperaturas también favorecen la formación de antioxidantes no polifenólicos (productos de la reacción de Maillard), algunos de los cuales podrían ser tóxicos y mutagénicos. Por lo tanto, el uso de altas temperaturas (sobre 100 o 150 °C) resulta en un bajo rendimiento de extracción de polifenoles pero en una alta recuperación de antioxidantes totales. Es así como, el extracto de orujo de uva obtenido a 200 °C muestra una mayor actividad y contenido antioxidante, pero un menor contenido de polifenoles que el extracto obtenido a 100 °C. Ambos extractos protegieron similarmente el crecimiento de células mamíferas (HL-60) bajo condiciones oxidantes mostrando una actividad comparable a la exhibida por Trolox (antioxidante de referencia). Sin embargo, el extracto obtenido a 200 °C presentó una alta citotoxicidad en ausencia del oxidante, probablemente debido a su alto contenido de compuestos de

Maillard. Estos resultados dejan en manifiesto la compleja y controvertida acción de los antioxidantes en los sistemas biológicos.

En conclusión, diferentes condiciones de extracción resultan en diferentes rendimientos de extracción, perfiles de antioxidantes y bioactividad. Una alta cantidad de polifenoles puede ser obtenida a temperaturas moderadas de extracción (100°C), mientras que a mayores temperaturas se logra una alta recuperación de antioxidantes totales debido a la formación nuevos antioxidantes (algunos potencialmente nocivos). Por otro lado, el efecto bioprotector de los extractos parece estar relacionado con el contenido de antioxidantes tanto polifenólicos como no polifenólicos, mientras que el contenido de compuestos de Maillard determina su citotoxicidad. Es por esto que los extractos obtenidos en procesos de extracción a alta temperatura, posiblemente no se pueden utilizar sin un proceso previo de purificación. Finalmente, el conocimiento presentado en esta tesis, junto con el generado en futuras investigaciones relacionadas, es esencial para controlar el proceso de EACP, mejorar la calidad de los extractos y definir los requisitos para procesos de purificación posteriores.

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Palabras Claves: Extracción con agua caliente presurizada, polifenoles, degradación térmica, productos de la reacción de Maillard, bioactividad.

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ABSTRACT

The supplementation of human diet with food rich in natural antioxidants prevents the development of several oxidation-related diseases. The most abundant antioxidants present in edible and non-edible plants as well as the waste generated from their processing are polyphenols. Phenolic compounds present biological activities that make them attractive for nutraceutical and medicinal applications: prevent the oxidative cell damage, act as antimutagens, anticarcinogens and antimicrobial agents, and are strong antioxidant compounds. From an economical point of view, recovering these bioactive phenolic compounds from agro-industrial by-products by clean, innocuous and inexpensive processes is crucial for the development and massification of nutraceutical products. The extraction with water at high temperature and pressure has arisen as a very attractive alternative to the use of organic solvents. Several physicochemical properties of water such as polarity, surface tension and viscosity can be manipulated through the change in temperature (maintaining water in its liquid state) to improve the solubility and diffusion rates of the target compounds. The pressurized hot water extraction (PHWE) process provides higher extraction yield and selectivities at shorter extraction times. However, the high temperatures also favour the degradation of thermolabile

compounds and the formation of new antioxidant compounds, which also could be harmful to health. In this thesis we determined the effects of the main PHWE parameters (temperature and time), on the extraction yield of several polyphenols classes and total antioxidants from two agro-industrial by-products (deodorized thyme and grape pomace). Then, we characterized two grape pomace extract obtained by PHWE at different temperatures (100 and 200 °C) in terms of chemical and biological antioxidant activity as well as their phenolic and non-phenolic antioxidant content.

In general, simple flavonoids (flavones, flavonols, flavanones and anthocyanins) and phenolic acids reach a maximum extraction yield at 100 °C. Higher temperatures (above 100 °C) and longer exposure times produce the degradation of these compounds. Polymeric flavonoids (condensed tannins) are maximally extracted at 150 °C evidencing high thermal stability (attributable to their polymeric structure). Higher temperatures also would favour the formation of non-polyphenolic antioxidants (Maillard reaction products), some of which could be toxic and mutagenic. Therefore, increasing extraction temperature (above 100 or 150 °C) results in a low extraction yield of polyphenols but in high recovery of total antioxidants. Thereby, the grape pomace extract obtained at 200 °C show higher antioxidant activity and content but lower polyphenols content than the extracts obtained at 100 °C. Conversely, both extracts protect similarly the growth of mammalian cells (HL-60) under oxidative conditions, showing an activity comparable to that exhibited by Trolox (antioxidant of reference). The extract obtained at 200 °C however showed a high cytotoxicity in the absence of oxidant, probably due to its high content of Maillard compounds. These results evidence the complex and disputed action of antioxidant on biological systems.

In conclusion, different extraction conditions result in different antioxidants recovery as well as antioxidants profile and bioactivity of the extracts. High polyphenols amount could be extracted at moderate extraction temperature (100 °C), whereas at high temperatures a high recovery of total antioxidant can be achieved due to the formation of new antioxidants (some of which are potentially harmful). The protective effect of the extracts on cell cultures under oxidative conditions appears to be

related to their content of both polyphenolic and non-polyphenolic antioxidants, whereas the Maillard compounds content determines their cytotoxicity. Consequently, the extracts obtained at high temperature processes such as PHWE possibly cannot be used without further purification processes. Therefore, to determine the contribution of the individual antioxidant compounds in the extract bioactivity is absolutely necessary to define the target compounds in further processes. Finally, the results presented in this thesis along with further related works could be useful to control the PHWE process, improve the quality of the extract and define the requirements for further purification processes.

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Key words: Pressurized hot water extraction, polyphenols, thermal degradation Maillard reaction products, bioactivity.

1 INTRODUCCIÓN

The growing concern for human wellbeing have generated an increase in the demand for polyphenols, secondary plant metabolites that exhibit antioxidant, radical-scavenging, antibacterial, antiviral, enzyme-inhibiting and antimutagenic properties (Quideau et al., 2011; Serrano et al., 2009). In fact, the demand for polyphenols (in terms of revenue) is expected to grow at a compound annual growth rate of 6.1% from 2012 to 2018, mainly due to the applications in functional foods and dietary supplements (Transparency Market Research, 2013). Hence, polyphenols are useful plant-derived compounds for the food and pharmaceutical industries, which are focused in generating bioactive natural products for human health and nutrition.

Obtaining polyphenols from plant material for their application in the food and pharmaceutical industries involves the use of adequate extraction processes. Traditionally, the extraction of polyphenols (and phytochemicals in general) has been mainly based on the use of Soxhlet devices and organic solvents. The Soxhlet technique repeatedly brings fresh solvent into contact with the solid matrix, do not require a filtration procedure and it is simple to operate (Wang and Weller, 2006). Nevertheless, traditional extraction methods such as Soxhlet employ large amounts of questionable solvents, are time consuming and have low selectivity and/or low extraction yield (Herrero et al., 2006a; Ollanketo et al., 2002). Besides, extraction with organic solvents might generate unsafe and potentially hazardous products for human consumption and the environment. Therefore, the use of clean, innocuous, environmentally friendly and efficient processes to extract polyphenols from plant material becomes crucial. Water-based extraction processes arise as a good option: water is non-flammable, non-toxic, readily available and an environmentally acceptable solvent. However, these processes are not widely used as an extraction method for plant materials because water is too polar to dissolve most organics, such as polyphenols (Ong et al., 2006). Indeed, given their nature and prevalence of non-ionic bonds in their structures, most typical

polyphenols present relatively low solubility in water at ambient conditions (Abou El Hassan et al., 2000; Boumendjel et al., 2003; Tommasini et al., 2004). Nonetheless, several physicochemical properties of water such as polarity, surface tension, viscosity and disassociation constant can be manipulated through the change in temperature to improve the effectiveness of the extraction process (Hawthorne et al., 2002; King et al., 2010). The pressurized hot water extraction (PHWE, also called superheated water extraction, subcritical water extraction, pressurized low polarity water extraction, hot compressed water extraction) process takes advantage of this feature, providing higher selectivities and shorter extraction times and avoiding the use of toxic organic solvents (King, 2000). Thus, PHWE appears as an attractive alternative to common organic extraction methods for obtaining polyphenols from plant materials.

1.1 Polyphenols: Key Bioactive Compounds

Polyphenols can be defined as secondary metabolites of relatively high molecular weight and diverse structural complexity which are synthesized by plants exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s) in response to different types of stress (hydric or saline) or aggressive factors (bacteria, fungi, virus, ultraviolet radiation, etc.) (Carrasco and Mizgier, 2013; Haslam, 1998; Quideau et al., 2011; Serrano et al., 2009). Polyphenols are widely distributed in the higher plant kingdom (Table 1.1); they are present in fruits, vegetables, herbs, spices, tea and wine (Djilas et al., 2009; Moure et al., 2001; Schieber et al., 2001).

Table 1.1 Sources of polyphenols. Information was collected from Moure et al. (2001), Schieber et al. (2001) and Dimitrios (2006).

Source	Polyphenols
Fruits	
Berries	Flavanols hydroxycinammic acids, hydroxybenzoic acids, anthocyanins
Cherries	Hydroxycinnamicacids, anthocyanins
Blackgrapes	Anthocyanins, flavonols
Citrus fruits	Flavanones, flavonols, phenolic acids
Plums, prunes, apples, pears, kiwi	Hydroxycinnamic acids, catechins
Vegetables	
Aubergin	Anthocyanins, , hydroxycinnamic acids
Chicory, artichoke	Hydroxycinnamic acids
Parsley	Flavones
Rhubarb	Anthocyanins
Sweet potato leaves	Flavonols, flavones,
Yellow onion, curly	Flavonols
Parsley	Flavones
Beans	Flavanols
Spinach	Flavonoids, *p*-coumaric acid
Flours	
Oats, wheat, rice	Caffeic and ferulic acids
TEAS	
Black, green	Flava-3-ols, flavonols

Source	Polyphenols
Alcoholic drinks	
Red wine	Flavan-3-ols, flavonols, anthocyanins
Cider	Hydroxycinnamic acids
Other drinks	
Orange juice	Flavanols
Coffee	Hydroxycinnamic acids
Chocolate	Flavanols
Herbs and spices	
Rosemary	Carnosic acid, carnosol, rosmarinic acid, rosmanol
Sage	Carnosol, Carnosic acid, lateolin, rosmanul, rosmarinic acid
Oregano	Rosmarinic acid, phenolic acids, flavonoids
Thyme	Thymol, carvacrol, flavonoids
Summer savory	Rosmarinic, carnosol, carvacrol, flavonoids
Ginger	Flavonoids and phenolic acids
By-products of fruit processing	
Apple pomace	Catechins, hydroxycinnamates, phloretin glycosides, quercetin glycosides, and procyanidins
Grape pomace	Anthocyanins, catechins, flavonol glycosides, phenolic acids and stilbenes
Citrus fruits pomace	Hesperidin, narirutin, naringin and eriocitrin

Source	Polyphenols
Mango seed	Gallic and ellagic acids, gallates, gallotannins and condensed tannin
Banana bracts	Anthocyanidins (delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin)
Kiwifruit pomace	Phenolic acids, flavanol monomers, dimers and oligomers, and flavonol glycosides
By-products of vegetable processing	
Onion residues	Quercetin glycosides
Olive residues	Hydroxytyrosol and derivatives
Red beet pomace	p-coumaric and ferulic acids
Potato peels	Phenolic acids (chlorogenic, gallic, protocatechuic and caffeic acids)
Others	
Durum wheat bran	Phenolic acids (chlorogenic, syringic, protocatechuic, gentisic, p-coumaric and vanillic acids)
Fraxinus ornus bark	Hydroxycoumarins
Corn bran hemicellulose	p-coumaric and ferulic acids
Oat hulls	Vanillic, p-coumaric and ferulic acids, catechol

Polyphenols show a great diversity of structures, ranging from rather simple molecules to polymers (Manach et al., 2004), with or without glycosylation and/or esterification. They may be classified in different groups as a function of the number of phenol rings that they contain and the structural elements that bind one ring to another. Four main groups can be distinguished (Fig. 1.1): (i) phenolic acids (hydroxybenzoic

acids, hydroxycinnamic acids, hydroxyphenylacetic acids, hydroyphenylpropanoic acids and hydroxyphenylpentanoic acids), (ii) flavonoids (anthocyanins, chalcones, dihydrochalcones, dihydroflavonols, flavanols, flavanones, flavones, flavonols, isoflavonoids), (iii) stilbenes, (iv) lignans and other polyphenols (alkylmethoxyphenols, alkylphenols, curcuminoids, furanocoumarins, hydroxybenzladehydes, hydroxybenzoketones, hydroxycinnamaldehydes, hydroxycoumarins, hydroxyphenylpropenes, methoxyphenols, naphtoquinones, tyrosols, phenolic terpenes and others) (Neveu et al., 2010).

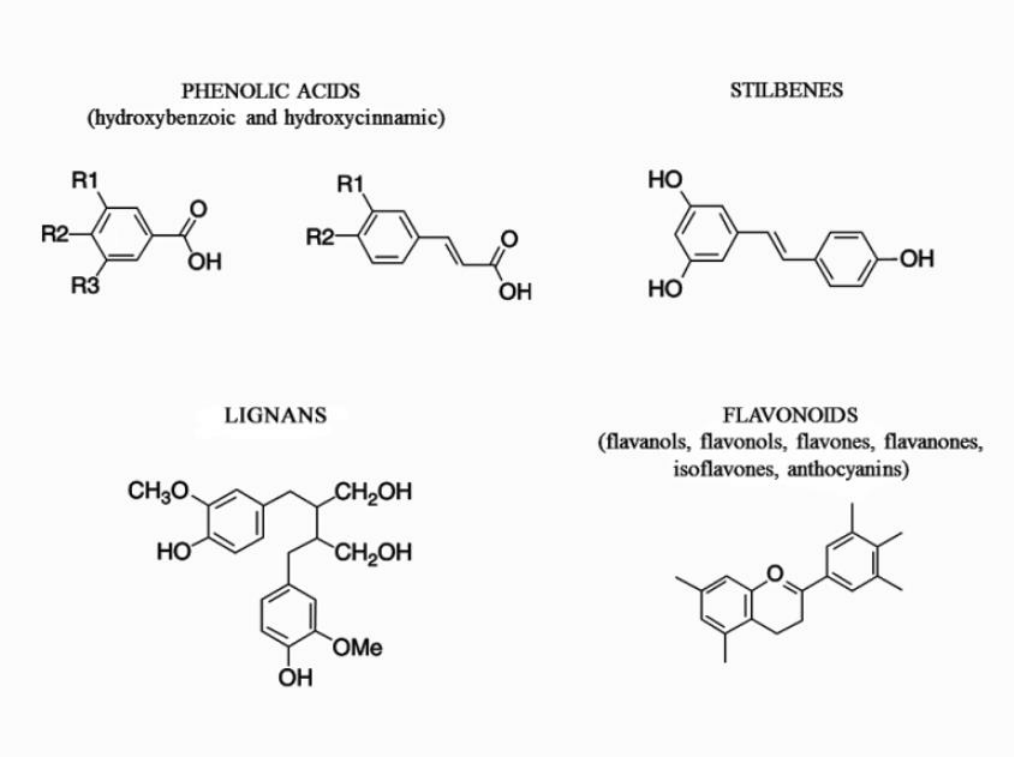

Figure 1.1 Main classes of polyphenols.

Polyphenols have the ability to complex strongly with metal ions and macromolecules such as polysaccharides and proteins (Haslam, 1998). Because of this ability, polyphenols present biological activities that make them attractive for

nutraceutical and medicinal applications (Collins, 2005; Hooper et al., 2008; Jensen et al., 2008; Manach et al., 2005; Ramassamy, 2006; Scalbert et al., 2005) and therefore for the food and pharmaceutical industries. Particularly, polyphenols play a key role in the inhibition of enzymes related to cardiovascular and neurodegenerative diseases, as well as cancer and diabetes (Quideau et al., 2011). There is evidence that they prevent oxidation of LDL-lipoprotein (Frankel et al., 1993; Fuhrman et al., 1997; Landbo and Meyer, 2001), platelet aggregation (Guerrero et al., 2007; Nardini et al., 2007), and oxidative cell damage (Paiva-Martins et al., 2009; Spormann et al., 2008). Additionally, polyphenols act as antimutagens and anticarcinogens (Cardador-Martinez et al., 2002; Kuroda and Hara, 1999), and antimicrobial agents (Chung et al., 1998; Yoda et al., 2004). Also, phenolic acids and flavonoids are the focus of recent studies because of their antioxidant properties, which are stronger than those of other commonly used natural and synthetic antioxidants (Chen and Ho, 1997; Miura et al., 2002).

Despite their demonstrated bioactive properties, the action of polyphenols on biological system is complex and disputed because it is affected by the bioavailability, metabolism and other biotransformations. The bioavailability is strictly related to their structures. The absorption and metabolism of these compounds depend on the degree of glycosylation and conjugation with other polyphenols. For example, non-glycosylated phenolic compounds may be absorbed directly into the small intestine (Manach et al., 2004). In turn, polyphenols present in the form of esters, glycosides, or polymers cannot be absorbed in the intestine. Polymers such as proanthocyanidins may have direct effects on the stomach (Pastene et al., 2009) and intestinal mucosa, protecting these tissues from oxidative stress or carcinogen action (Manach et al., 2005). To become absorbed in the intestine, esterified, glycosylated and polymeric polyphenols require to be hydrolyzed by enterocytes enzymes or through the action of colonic microbiote (Carrasco and Mizgier, 2013). The antioxidant activity of polyphenols is arguable: although studies in cell line cultures have shown that polyphenols are able to reduce oxidative stress and activate the antioxidant response of the cells, they also could decrease the cell viability and proliferation and induce the cell apoptosis by acting as prooxidants and generating free

radicals (Xia et al., 2010). The effects of the polyphenols in cell cultures (either protective antioxidant or prooxidant/cytotoxic) will depend on several factors such as their concentration, their ability to oxidize, their lipophilicity, the content of other antioxidants and metals, and the oxidative stress level of the cell culture (Fujii et al., 2006; Halliwell, 2008; Hou et al., 2004; Sergediene et al., 1999; Surh et al., 1999; Wei et al., 1995). Therefore, to safely use polyphenols as bioactive compounds it is necessary to carefully study aspects such as the dosage, the delivery vehicle, as well as the nutritional and health history and the microbiota characteristics of the target population (Manach et al., 2004).

1.2 Pressurized Hot Water Extraction

As it was mentioned before, several physicochemical properties of water such as polarity, surface tension, viscosity, diffusivity and dissociation constant can be manipulated through the change in temperature. For instance, water polarity is dramatically reduced while increasing temperature due to the breakdown of the hydrogen bonds that form the water structure. The dielectric constant (ε, measure of polarity) of water at 25 °C is approximately 80 (extremely polar), but at 250 °C, the water in liquid state reach values of ε between 25 and 27, which are comparable to the values of methanol (ε = 33) and ethanol (ε = 24) at 25 °C (Ong et al., 2006; Teo et al., 2010). Also, the viscosity and surface tension of water decrease with the temperature (Hawthorne et al., 2002) (Fig. 1.2). On the contrary, high temperatures increase the diffusivity and dissociation constant of water (Bandura and Lvov, 2006; Holz et al., 2000).

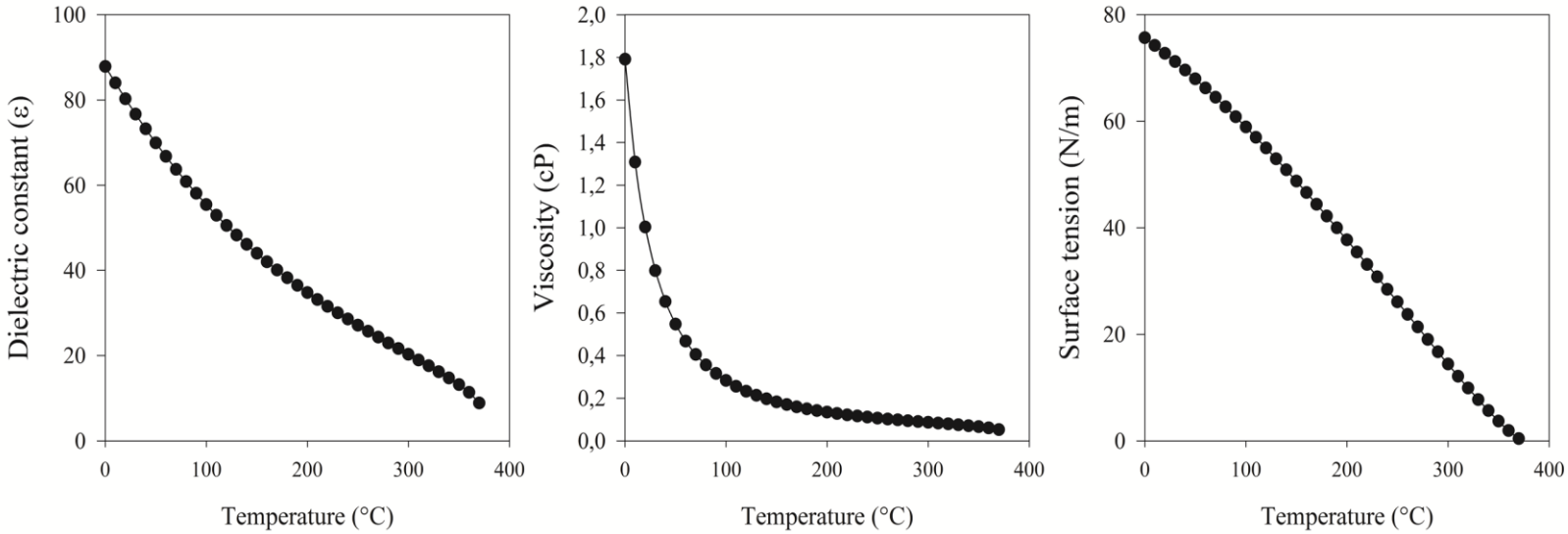

Figure 1.2 Decrease of the dielectric constant, viscosity and surface tension of water with temperature (data obtained from Beaton et al. (1987)).

Pressurized hot water extraction (PHWE) takes advantage of the features described before by raising the temperature to values within 100 °C and 374 °C, while applying sufficient pressure to maintain water in a liquid state. Both temperature and pressure play a significant role in the disruption of surface equilibrium decreasing the activation energy required for the desorption process (Clifford, 2005; Hawthorne et al., 2002; Ong et al., 2006; Smith, 2006). Besides, the decreases in viscosity and surface tension of water enhance solvent penetration and wetting of the matrix (Teo et al., 2010). In addition, higher diffusivities favor mass-transfer rates by disrupting intermolecular forces (i.e. van der Waals forces, hydrogen bonds and dipole attractions) (Herrero et al., 2013). Moreover, the changes in the dissociation constant of water would facilitate the release of interest compounds from vegetable matrices since pressurized hot water could act as an acid and/or base catalyst for reactions, such as hydrolysis of lignocellulosic material (Yu et al., 2007). Despite all the advantages of using PHWE, the target compounds may also be subjected to thermal decomposition and hydrolytic attack (Ju and Howard, 2005; Vergara-Salinas et al., 2013; Vergara-Salinas et al., 2012).

Typically, PHWE processes are carried out in a system such as that showed in Fig. 1.3. In this system, the extraction cell containing the sample (e.g. the plant matrix) is filled with water, heated to the desired temperature and pressurized to maintain water as a liquid; then the extraction occurs (Raynie, 2006). A pump is used to pressurize and transport water from a container, a preheating coil brings the water up to the operating temperature before entering into the extraction cell, an oven maintains the temperature in the extraction cell, a valve or restrictor is used to generate the required back pressure and a trapping vial collects the extract. Optionally, the system can include a nitrogen circuit to purge the cell after the extraction and a coil to cool the water exiting the oven (Herrero et al., 2013; Teo et al., 2010). The extraction process is performed at pressures between 10 and 60 bar (typically 50 bar) and temperatures above the boiling point of water (up to 200-250°C).

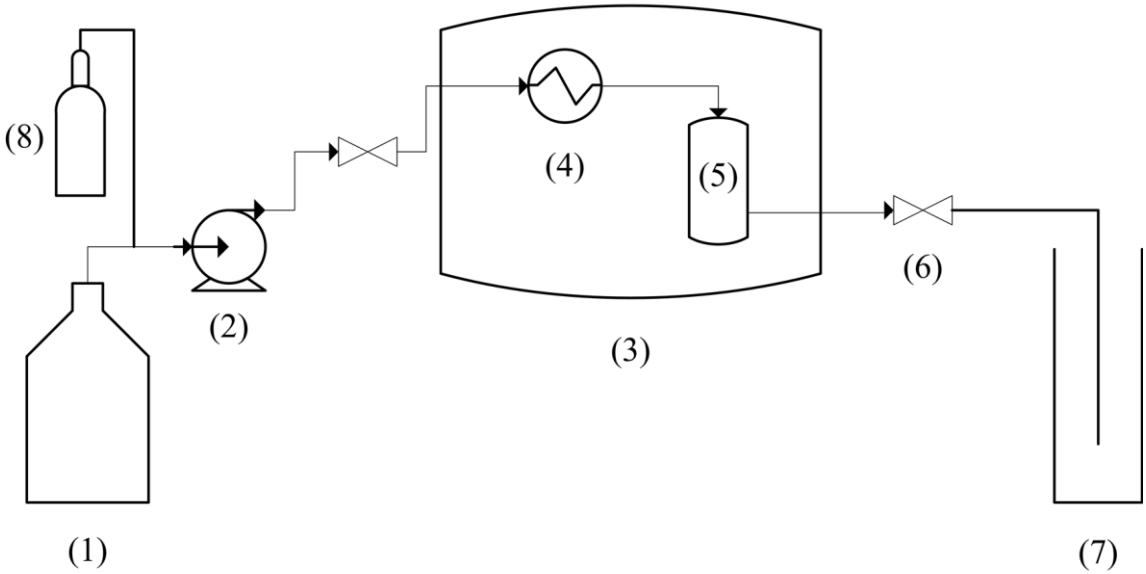

Figure 1.3 Typical system for PHWE processes.
It consists of: (1) a water container; (2) a water pump; (3) an oven; (4) a preheating coil; (5) an extraction cell; (6) a restrictor or valve; (7) a trapping vial; and (8) a modifier pump or N_2 container (optional) (adapted from Herrero et al. (2013))

The extraction mechanism of PHWE involve four sequential steps: (i) the desorption of solutes from the matrix; (ii) the diffusion of the extraction fluid into the matrix; (iii) the partition of the solutes from the matrix into the extraction fluid; and (iv) the elution of the solutes from the extraction cell to the collection vessel. Therefore, the process may be controlled by several phenomena such as solubility, sorption equilibria and diffusion, whose incidence depends on the extraction modes and conditions (Mustafa and Turner, 2011; Teo et al., 2010). The extraction can be performed in static or dynamic mode. In static mode, the most frequently used, the raw plant material is kept in the extraction cell with a given volume of water during a certain period of time at fixed conditions of pressure and temperature. The extraction efficiency strongly depends on the partition coefficient of the target compounds in the water/plant matrix system and

the solubility of these compounds in water (which is analyzed later on this chapter). Therefore, the extraction time and cycles of extraction (replacement of the solvent to deplete the matrix) must be carefully optimized. The extraction time must allow the saturation of water with the target compounds and minimize the exposure of the compounds to high temperature. Generally, in static PHWE, the equilibrium is quickly reached (5-30 min) (Mukhopadhyay and Panja, 2010).

In dynamic extraction mode, the pressurized hot water passes continuously through the matrix kept in the extraction cell (it can be called a semi-continuous process). The phase equilibria might be displaced and the efficiency of the extraction process might be increased (Herrero et al., 2013). Besides extraction time, the flow rate of water (usually within the range of 1 to 1.5 mL/min) is another important parameter for the optimization of PHWE operating in a dynamic mode (Teo et al., 2010). If the flow rate is large enough, the diffusion of the solute within the matrix (internal mass transfer) controls the extraction rate, and therefore, further increase in the flow rate would have little effect on the extraction rate (Pronyk and Mazza, 2009). If the flow rate is low enough, the removal of the solute from the surface of the matrix decreases, and the extraction is controlled by external mass transfer from solid to the solvent. In this case, an increase in the flow rate would result in an increase of the extraction rate of the solute (Pronyk and Mazza, 2009). Despite its advantages, dynamic PHWE generates more diluted extracts than those obtained in static mode, increasing post-treatment costs (Herrero et al., 2013).

PHWE has the advantages of being relatively inexpensive, minimizes or totally avoids the use of objectionable solvents, and provides a facile means for supplying a wide source of concentrated phytochemicals for use in food formulation, dietary supplements and phyto-pharmaceutical applications (King and Grabiel, 2007). Thus, several reviews articles (Herrero et al., 2013; Herrero et al., 2006a; Mendiola et al., 2007; Mukhopadhyay and Panja, 2010; Mustafa and Turner, 2011; Ong et al., 2006; Smith, 2002; Teo et al., 2010; Wiboonsirikul and Adachi, 2008), book sections (Cacace and Mazza, 2006a; Clifford, 2005; Srinivas and King, 2010) and patents (Hawthorne et

al., 2002; King and Grabiel, 2007) that cover or are completely dedicated to the PHWE of phytochemicals have been published. Even, articles about design, performance improvements and scaling up of extraction process of bioactive compounds at high temperature and pressure (Ibañez et al., 2009; King and Srinivas, 2009; Monrad et al., 2012; Pronyk and Mazza, 2009; Rodríguez-Meizoso et al., 2012; Srinivas et al., 2009) have been published recently demonstrating the increasing interest in the development of these green extraction processes. Additionally, pressurized hot water have been used for other different applications such as selective separation of organic compounds from liquids, selective separation of organic compounds using adsorbent phases, improvement of reactions controlling the dissociation constant, cleaning of waste water and oxidative degradation compounds for soil remediation (Coym and Dorsey, 2004; Fields et al., 2001; Hawthorne et al., 2000; Kuosmanen et al., 2002; Ong et al., 2006; Soltanali et al., 2009; Yang et al., 1995; Yesodharan, 2002).

1.3 Pressurized Hot Water Extraction of Polyphenols

In this section, the key parameters for designing and optimizing the PHWE processes of polyphenols (e.g. aqueous solubility and temperature) and their effects on extraction yields and extracts activity and composition are analyzed. Also, operation modes and equipment for the PHWE of polyphenols are described. Finally, the application of PHWE to obtain different polyphenols from plant sources is discussed and compared to traditional solvent extraction methods.

1.3.1 Solubility of polyphenols in pressurized hot water

As mentioned before, PHWE techniques enable the use of liquid water at temperatures above its atmospheric boiling point. This characteristic enhances the

aqueous solubility of polyphenols and therefore the efficiency of the extraction process. Thus, aqueous solubility is a key parameter for designing and optimizing PHWE processes. Particularly, aqueous solubility controls the first stage of the PHWE process, where the external mass transfer occurs. In this first stage, the solute (mainly present on the surface of the material being processed) is freely available to be removed as long as the maximum solubility of the solute within the solvent (in our case water) has not been reached (Pronyk and Mazza, 2009).

High pressures used in PHWE have practically no effect on the aqueous solubility, since water in that conditions (as well as other liquids in subcritical conditions) is largely incompressible (Smith, 2002). Solubility in pressurized hot water is primarily regulated by temperature, which weakens the intermolecular hydrogen bonds within water and reduces its dielectric constant (and therefore its polarity). At ambient conditions, the dielectric constant of water is 80 and it can reach a value of 27 at 5 MPa and 250°C (Yang et al., 1995), which is close to those of organic solvents such as ethanol and methanol at 25°C. Hence, water becomes a solvent suitable to dissolve many hydrophobic compounds (aqueous solubility increases) as temperature increases above 100°C (at adequate high pressures) (Carr et al., 2010).

Since polyphenols have a tendency to thermally degrade at high temperature, the measurement of their aqueous solubilities above the boiling point of water is difficult (Srinivas and King, 2010). Besides, solubility experiments can be unmanageable (i.e. time consuming and expensive) because it is extremely difficult to isolate the polyphenols at the levels of required purity. Usually, the polyphenol must first be extracted from plant tissues, then purified and isolated from other undesired compounds and finally, characterized in terms of its major molecular features. Although this entire procedure is widely covered in the literature (Ignat et al., 2011; Khoddami et al., 2013; Mueller-Harvey, 2001; Naczk and Shahidi, 2004; Santos-Buelga and Williamson, 2003; Serrano et al., 2009) until now no standard or completely satisfactory methodologies to obtain the pure polyphenols are available and their effectiveness strongly depends on their molecular complexity. For instance, extraction, purification and isolation of tannins

are quite dependent on their degree of polymerization (highly-polymerized tannins are difficult to analyze) (Serrano et al., 2009). Therefore, scarce data on the solubility of polyphenols in PHW can be found in the literature. For instance, Srinivas et al. (2010a, 2010b) measured the solubility of two phenolic acids (gallic acid hydrate and protocatechuic acid), a flavan-3-ol ((+)-catechin hydrate) and flavonols (quercetin and its dehydrate) in PHW using a dynamic flow apparatus coupled to a HPLC system designed to avoid thermal degradation of polyphenols. Results showed that the solubility of these polyphenols in PHW increase exponentially with temperature. Also, some data on the aqueous solubility of polyphenols (particularly those with simpler molecular structures) at atmospheric conditions can be found in literature. Noubigh et al. (2007b) and Queimada et al. (2009) have determined the aqueous solubility of two particular polyphenols (vanillin and tyrosol, respectively) at temperature ranges bellow the normal boiling point and atmospheric pressure using standard shake-flask methods coupled to UV spectrophotometry or HPLC methods. Furthermore, several authors have measured the aqueous solubility of different phenolic acids such as gallic (Daneshfar et al., 2008; Lu and Lu, 2007; Mota et al., 2008; Noubigh et al., 2008), vanillic, ferulic (Noubigh et al., 2007a), trans-cinnamic, caffeic (Mota et al., 2008), protocatechuic, syringic, o-coumaric, ellagic (Queimada et al., 2009) and salicylic acids (Matsuda et al., 2008; Nordström and Rasmuson, 2006; Shalmashi and Eliassi, 2007), using similar conditions and methods. In all these cases, solubility of polyphenols in water at atmospheric conditions also increases with temperature. Other methods can be found in literature to determine the experimental solubility of polyphenols at fixed conditions. For instance, Haslam (1996) compared the aqueous solubility of two similar tannins, castalagin and vescalagin, with that of the tannin *β*-1,2,3,4,6-penta-*O*-galloyl-D-glucose in terms of their octanol/water partition coefficient (*K* [octanol/water]) at 25°C and 1 atm. He found that castalagin and vescalagin were more water soluble, since they had a *K* [octanol/water] = 0.1, lower than the value for *β*-1, 2, 3, 4, 6-penta-*O*-galloyl-D-glucose (*K* [octanol/water] = 32). Tanaka et al. (2000) estimated the aqueous solubility of a polymeric tannin fraction extracted from Paeoniae Radix, an important crude drug in

Chinese traditional medicine. They found that the polymeric fraction was 25% less soluble in water than in a 50% methanol solution by comparing the peak areas obtained after a HPLC analysis at 28°C.

1.3.2 Key parameters for optimizing the PHWE of polyphenols

In the PHWE of polyphenols, the temperature and extraction time mainly define the total extraction yield, polyphenol content and composition and antioxidant activity of the extracts, which are strictly related to the efficiency of the process. PHWE of phenolic compounds is a complex process where appears several phenomena such as thermal degradation, selective extraction of polyphenols and the formation of neo-antioxidant and toxic compounds (Martins et al., 2000; Yilmaz and Toledo, 2005), which are highly dependent on extraction temperature and exposure time at that temperature (Duan et al., 2009; Plaza et al., 2010c, 2010d; Rodriguez-Meizoso et al., 2006; Srinivas et al., 2011).

Since polyphenols are compounds sensitive to high temperatures, especially above 100 °C (Nakamura et al., 1998; Palma et al., 2001; Srinivas et al., 2011), the extraction yield of these specific compounds during PHWE will be determined by both their solubility and their degradation rate at the fixed operating temperature and time. Explaining it simply, the polyphenols solubility in water and then their extraction increases as the temperature and time increase up to certain range of conditions at which the thermal degradation becomes predominant. For instance, Kumar et al. (2011), reported that the highest extraction yield of quercetin-3-galactoside, kaempferol and isorhamnetin from seabuckthorn leaves was at 150 °C (temperature range of study from 50 to 200°C). Rangsriwong et al. (2009) studied the PHWE of gallic acid, ellagic acid, and corilagin from *Terminalia chebula* Retz fruits between 120 and 220°C. The extraction of gallic acid and ellagic acid increased with the extraction temperature up to a maximum at 180°C, whereas the highest amount of corilagin was recovered at 120°C.

In the PHWE of phenolics from red grape skin, Ju and Howard (2005) reported that increasing the temperature from 100 to 110°C produced an increase in the extraction of anthocyanins, although extraction temperatures above 110°C resulted in lower contents of individual and total anthocyanins. The extraction of anthocyanin-tannin polymeric pigments through PHWE increases with temperature, especially above 100°C. In the PHWE of tannins from grape seeds, increasing the extraction temperature caused an increase in the tannin extraction yield, peaking at 150°C (García-Marino et al., 2006). Although tannins are more thermo-stable than most of the other polyphenols, may be due to their polymeric structure (Larrauri et al., 1997), extraction temperatures above 150°C may cause their degradation (Gaugler and Grigsby, 2009).

Due to the different polarities, polyphenols could be also extracted selectively by PHWE. Rodriguez-Meizoso and coworkers studied the selective PHWE of polyphenols from oregano leaves (*Origanum vulgare* L.) at several temperatures (Rodriguez-Meizoso et al., 2006). The extracts obtained at different temperatures were analyzed by HPLC-DAD. The identified compounds were from the flavanone and dihydroflavonol subclasses manly. Flavonols were also found in the form of quercetin and, in lesser amount, flavones. At the lowest temperature, the more polar compounds, flavanones and dihydroflavonols were preferentially extracted. Whereas, a temperature increase up to 200 °C favored the extraction of the less polar compounds by several orders of magnitude, resulting in such compounds being the major constituents of these extracts. Ibañez et al. (2002) also studied the selective recovery of antioxidants compounds from rosemary by PHWE. Results indicate high selectivity of PHW for the most active compounds of rosemary: carnosol, rosmanol, carnosic acid, methyl carnosate, cirsimaritin and genkwanin. Depending on the extraction temperature, the extracts showed different compound profiles (Fig. 1.4). Carnosol, rosmanol and genkwanin were preferentially extracted at 100°. At 200 °C, the extraction of carnosic acid, methyl carnosate and cirsimaritin was favored.

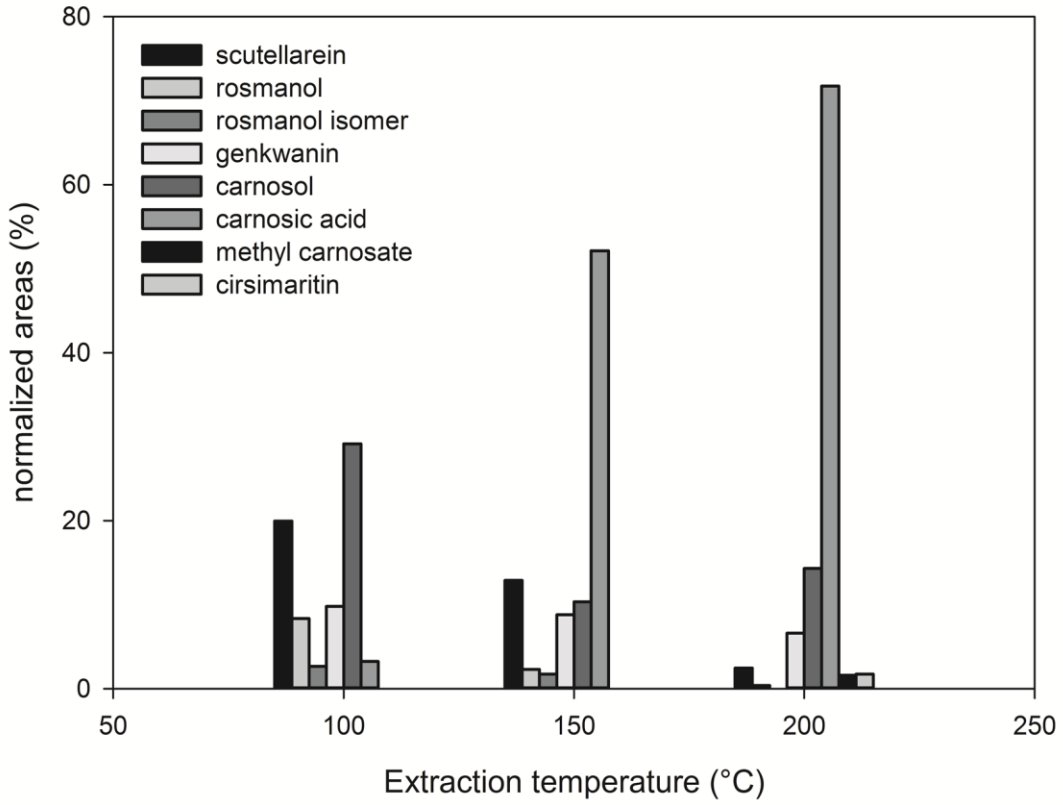

Figure 1.4 Selective recovery of antioxidant compounds from rosemary by PHWE (data adapted from Ibañez et al. (2002)).
Normalized areas were obtained by GC-MS analysis.

Other types of antioxidants compounds can also be found in the extracts obtained at high temperatures, particularly products from Maillard, thermoxidation and caramelization reactions. For instance, Plaza et al. (2010a) found that the formation of Maillard products (fluorescent advanced glycated end products) and browning products (e.g. melanoidins) are significantly favored at 200 °C compared to 100 °C. Kulkarni et al. (2008), who studied the PHWE of antioxidants from *Eucaliptus grandis*, found that the main antioxidant compounds obtained in the extracts were pyrogallol, 5-hydroxymethyl-2-furaldehyde (HMF) and 4,4,6,6-tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid, which are not originally present in the untreated plant. Apparently

these compounds were formed through degradation reactions generated by the high reactivity of water at high temperatures. In the PHWE of pomegranate, He et al. (2012) also studied the formation of Maillard reaction, particularly the content of HMF in the extracts. The results indicated that intermediate Maillard reaction products increased with temperature up to a maximum at 220 °C and decreased when temperature was higher than 240 °C. Whereas, the formation of final Maillard reaction products increased constantly with the extraction temperature. In particular, the HMF content in the extracts increased more than 10 times when the extraction temperature was elevated from 100 to 220 °C. At temperatures higher than 220 °C the HMF content decreased due to its degradation.

Temperature and extraction time also affect the activity of the polyphenolic extracts obtained from plant matrices by PHWE. Specifically, both parameters have a strong effect on the antioxidant activity of the extracts. In general, antioxidant activity increases with the extraction temperature (Baek et al., 2008; Herrero et al., 2010; Kumar et al., 2011; Rodriguez-Meizoso et al., 2006), mainly because the solubility and diffusion rate of several polyphenols is enhanced. However, at high temperatures the formation of antioxidant compounds from Maillard, thermoxidation and caramelization reactions occurs. For instance, He et al. (2012) observed that the HMF behavior coincides with trend of antioxidant activity (Fig. 1.5), showing the significant contribution of the new antioxidant formed at high temperatures to the antioxidant activity of the extract. Additionally, products from the thermal decomposition of polyphenols (e.g. caffeic acid) and lignocellulose during the PHWE of various plant matrices have revealed high antioxidant activity (Chen and Ho, 1997; Guillot et al., 1996; Hendriks and Zeeman, 2009; Wahyudiono et al., 2008). Although Maillard reaction products strengthen the antioxidant activity of the extracts obtained by PHWE, they can also be toxic, mutagenic and carcinogenic (Husøy et al., 2008; Martins et al., 2000; Yilmaz and Toledo, 2005). Therefore, care should be taken when maximizing the antioxidant activity of plant extracts obtained through PHWE. Plaza et al. (2013) made an interesting study evaluating the effect of the temperature and time on the flavonols

recovery, antioxidant activity and formation of Maillard reaction products in the PHWE of apple by-products. By using the response surface methodology they determined the optimum condition for flavonols extraction (120 °C and 3 minutes) as well as the condition which maximizes the antioxidant activity and minimizes the formation of Maillard reaction products (125 °C and 3 minutes).

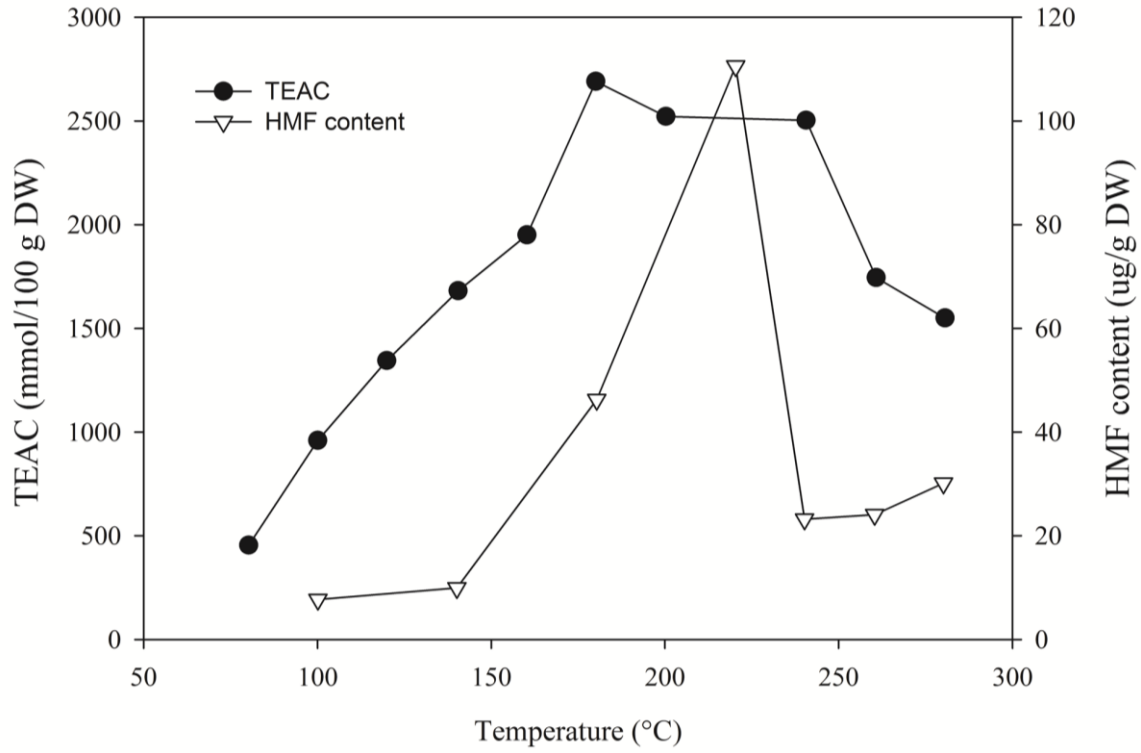

Figure 1.5 Relationship between the increase in the antioxidant activity (Trolox equivalent antioxidant capacity, TEAC) and the formation of hydroxymethylfurfural (HMF, a Maillard reaction product) in the pressurized hot water extraction of polyphenols from pomegranate (data adapted from He et al. (2012)).
DW: dry extract.

Additionally, since increasing the extraction temperature also causes an increase in the capacity of water to solubilize and hydrolyze several components of plant

materials, a great amount of diverse compounds can be released during PHWE (Herrero et al., 2006b; Herrero et al., 2010; Ong et al., 2006; Plaza et al., 2010a). At temperatures of 160 °C and higher, pressurized hot water is able to solubilize hemicellulose (Ingram et al., 2009) and lignin (Liu and Wyman, 2003). Moreover, during a water-based thermal process, part of the hemicellulose is hydrolyzed and forms acids. These acids are assumed to catalyze hydrolysis of remaining hemicelluloses (Gregg and Saddler, 1996). The hydrolysis of lignocellulosic material in PHWE of polyphenol-rich materials, which is usually overlooked, may contribute to the release of phenolic cell wall-associated compounds (Pérez-Jiménez and Torres, 2011). Also, the lignocellulose hydrolysis contribute to obtain sugars, a raw material for several processes, e.g. conversion to biofuels (Yu et al., 2007).

1.3.3 Equipment and modes of operation in the PHWE of polyphenols

The system described before in Fig. 1.3 has been widely applied for the PHWE of polyphenols from plant material using laboratory-scale equipment. For instance, homemade units have been used for the PHWE of flavonoids from aspen knotwood (Hartonen et al., 2007) and phenolic compounds from potato peels (Singh and Saldaña, 2011). However, most of the applications are based on commercially-available Accelerated Solvent Extractors (ASE) developed by Thermo Scientific Dionex (Salt Lake City, UT, USA), which have been used for obtaining phenolic extracts from agroindustrial by-products (Co et al., 2009; Herrero et al., 2011; Ko et al., 2011) and herbs (Dawidowicz et al., 2009; Kumar et al., 2011; Vergara-Salinas et al., 2012). Although PHWE is a straightforward scalable process from data gathered in small-scale experiments (Pronyk and Mazza, 2009), no applications were found in the literature for the pilot-scale and industrial-scale PHWE of polyphenols. However, apparatus designed for other industrial applications, such as those used for the remediation of contaminated soils (Lagadec et al., 2000) could be adapted and used for that purpose. Also, specialized

companies such as Thyssenkrupp-Uhde (Dortmund, Germany), Chematur Technologies (Karlskoga, Sweden), Flavourtech Pty. Ltd. (Griffith, Australia) and Critical Processes Ltd (North Yorkshire, UK) could modify their high pressure technologies or develop new ones for the industrial-scale PHWE of polyphenols. Particularly, an extraction pilot plant located at Critical Processes Ltd. and described in Clifford (2005) is an excellent approach to develop industrial PHWE processes.

PHWE of polyphenols is mainly carried out in a static manner. If phase equilibria is reached in this mode (i.e. the polyphenol of interest is completely solubilized in water), the efficiency of the process will not increase beyond this point and the optimization of the extraction time becomes fundamental (Herrero et al., 2013). However, several batch extraction cycles with replacement of pressurized hot water can be used to surpass this drawback. Most of the works already cited in this chapter (which fundamentally uses laboratory-scale equipment) deals with this static extraction mode. For instance, Plaza et al. (2013) have extensively studied the static PHWE of several antioxidant polyphenols from apple by-products. Dynamic extraction mode has been also used for the PHWE of polyphenols, but to a lesser degree. For instance,Ho et al. (2008) analyzed the dynamic PHWE of lignans from flaxseed meal. They reported that internal mass transfer was the controlling factor in the process since changing the flow rate had no effect on the extraction rate. Rangsriwong et al. (2009), who studied the dynamic PHWE of polyphenols from *Terminalia chebula* Retz. fruits, demonstrated that the extraction rate of the target compounds was influenced by external mass transfer given that the extraction increased with an increase in the volumetric flow rate from 2 to 3 or 4 mL/min. One of the main advantages of using dynamic PHWE instead of the static mode would be related to the thermal stability of polyphenols: the continuous flow of water through the extraction cell of a dynamic system contributes to avoid the thermal degradation of polyphenols (Ibañez et al., 2012).

Commercial laboratory-scale equipment commonly used for the PHWE of polyphenols (Dionex ASE) can only be operated in static (batch) mode. In turn, a homemade equipment can be used in a flexible manner to perform both dynamic and

static extractions (Ibañez et al., 2012). A good example of a homemade dynamic equipment is the semicontinuous system developed by Monrad et al. (2012) to extract several polyphenols from grape pomace. Unlike the typical system showed in Fig. 1.3, the extraction cell is placed outside the oven to avoid excessive exposure of the sample to high temperature and hence prevent its decomposition. Continuous PHWE processes for polyphenols are slightly covered in literature. For instance, Soto Ayala and Luque de Castro (2001) successfully developed a continuous PHWE system to isolate essential oils rich in phenolic terpenes from ground oregano leaves. Technologies from the aforementioned companies (Thyssenkrupp-Uhde, Chematur Technologies, Flavourtech Pty. Ltd. and Critical Processes Ltd.) can be adapted to develop a continuous PHWE process. For instance, the Integrated Extraction System (IES) from Flavourtech Pty. Ltd., which has been used to obtain flavors and extracts from tea leaves and coffee beans at normal conditions, is an interesting alternative. IES is an equipment of five modules (slurry preparation, flavor extraction, clarification, washing and concentration) that pre-treats the raw plant material by milling and mixing it with water to generate a slurry, performs the extraction of volatile flavor compounds from the slurry using a countercurrent water flow, and clarifies and concentrates the flavor-stripped slurry to obtain the desired extract.

1.3.4 PHWE of different polyphenols from plant sources

In the last years, there has been an increase in the studies on PHWE of phytochemicals from plants and fruits, especially polyphenols. Particularly, aromatic herbs and agro-industrial by-products have been extensively explored as sources of polyphenols (Table 1.2). PHWE of essential oils rich in phenolic terpenes, flavonoids, phenolic acids and condensed tannins are discussed in next sub-sections.

Table 1.2 Main references on PHWE of polyphenols published during the last 15 years. Details about sources of extraction and their principal compounds (fundamentally polyphenols) are included.

Source	**Principal compounds**	**Reference**
Herbs, spices and shrubs		
Clove buds (*Syzygium aromaticum*)	Eugenol and eugenyl acetate	(Rovio et al., 1999)
Hops samples (*H. lupulus* L.)	Prenylflavonoids	(Gil-Ramírez et al., 2012)
Gastrodia elata Blume	Gastrodin and vanillyl alcohol	(Teo et al., 2008)
Licorice roots (*Glycyrrhiza uralensis* Fisch)	Glycyrrhizin, glycyrrhetic acid and liquiritin	(Baek et al., 2008)
Milk thistle *(Silybum marianum)*	Taxifolin, silichristin, silidianin, silibinin, and isosilibinin	(Duan et al., 2009)
Oregano leaves (*Origanum onites*)	Borneol, terpinen-4-ol and carvacrol	(Ozel and Kaymaz, 2004)
Oregano leaves (*Origanum vulgare*)	Phenolic antioxidants flavanones, dihydroflavonols, favonols and flavones	(Rodriguez-Meizoso et al., 2006)
Peppermint (*Mentha piperita*) and Savory *(Satureja hortensis)*	Oxygenated and non-oxygenated flavour and fragrance compounds	(Kubátová et al., 2001)
Rosemary leaves	Oxygenated fragrance and flavor compounds	(Basile et al., 1998)

Source	Principal compounds	Reference
(*Rosmarinus officinalis*)		
	Carnosol, rosmanol, carnosic acid, methyl carnosate, cirsimaritin and genkwanin	(Ibañez et al., 2002)
	Rosmarinic acid, carnosic acid, caffeic acid, and carnosol (among others)	(Herrero et al., 2010)
	Total phenolic compounds Carbohydrates Proteins Maillard reaction products (melanoidins and advanced glycation end products)	(Plaza et al., 2010a)
	Antioxidants	(Rodríguez-Meizoso et al., 2012)
Sage (*Salvia officinalis*)	Rosmarinic and carnosic acids, carnosol and methyl carnosate	(Ollanketo et al., 2002)
Seabuckthorn leaves (*Hippophae rhamnoides*)	Quercetin-3-galactoside, kaempferol, and isorhamnetin	(Kumar et al., 2011)
Tea leaves (*Camellia sinensis*)	Catechin and epicatechin	(Piñeiro et al., 2004)
Thyme leaves (*Thymus vulgaris*) and Verbena (*Verbena officinalis*)	Total phenolic compounds Carbohydrates Proteins Maillard reaction products (melanoidins and advanced glycation end products)	(Plaza et al., 2010a)
Fruits, seeds and vegetables		
Bitter melon fruits	Catechin, gallic acid, gentisic acid and	(Budrat and

Source	Principal compounds	Reference
(*Momordica charantia*)	chlorogenic acid	Shotipruk, 2009)
Defatted flaxseed meal (*Linum usitatissimum*)	Lignans	(Ho et al., 2007)
Defatted rice bran (*Oryza sativa*)	Total phenolics compounds Carbohydrates Proteins Furfural	(Wiboonsirikul et al., 2007)
	Gallic acid, caffeic acid, *p*-coumaric acid and ferulic acid	(Fabian et al., 2010)
Defatted soybean flakes	Isoflavones	(Li-Hsun et al., 2004)
Flaxseeds (*Linum usitatissimum*)	Lignans and other phenolics	(Cacace and Mazza, 2006b)
Onion	Quercetin-3,4-O-diglucoside, (2) quercetin-3-glucoside and quercetin-4-O-glucoside	(Andersson et al., 2012)
Soybean seeds	Isoflavones	(Luthria et al., 2007)
Terminalia chebula Retz. fruits	Gallic acid, ellagic acid, and corilagin	(Rangsriwong et al., 2009)
Agroindustrial by-products		
Apple by-products	Polyphenols: 5-caffeoylquinic acid, hyperoside, isoquercitrin, reinutrin, phloridzin, avicularin, quercitrin and quercetin.	(Plaza et al., 2013)
	Maillard reaction products (melanoidins, furfural, and hydroxymethylfurfural)	

Source	Principal compounds	Reference
Aspen knotwood (*Populus tremula*)	Naringenin, dihydrokaempferol, naringin and taxifolin	(Hartonen et al., 2007)
Citrus peels (*Citrus unshiu*)	Hesperidin and narirutin	(Cheigh et al., 2012)
Citrus pomace (*Citrus unshiu*)	Polymethoxylated flavones: sinensetin, nobiletin, and tangeretin	(Kim et al., 2009)
Flooded gum leaves (*Eucalyptus grandis*)	Pyrogallol, 5-hydroxymethyl-2-furaldehyde, and 4,4,6,6-tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid	(Kulkarni et al., 2008)
Grape pomace (*Vitis vinífera*)	Total phenolic compounds Total flavonoids	(Aliakbarian et al., 2012)
	Anthocyanins and procyanidins	(Monrad et al., 2012)
Grape seeds (*Vitis vinifera*)	Catechin and epicatechin	(Piñeiro et al., 2004)
	Catechins and proanthocyanidins	(García-Marino et al., 2006)
Grape skin (*Vitis vinifera*)	Anthocyanins	(Ju and Howard, 2003)
	Anthocyanins, flavonols, hydroxycinnamates, phenolic acids	(Ju and Howard, 2005)
	3-O-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin	(Šťavíková et al., 2011)
Mango leaves (*Mangifera indica*)	Mangiferin and quercetin 3-β-d-glucoside	(Fernández-Ponce et al., 2012)

Source	Principal compounds	Reference
Olive leaves (*Olea europaea*)	Secoiridoids, tyrosols, hydroxycinnamic acid derivatives and flavonoids	(Herrero et al., 2011)
Onion skins	Quercetin, quercetin-4-O-glucoside kaempherol, isorahmnetin, and rutin	(Ko et al., 2011)
Orange peel	Anilines, phenols and flavones	(Lamm and Yang, 2003)
Pomegranate peels (*Punica granatum*)	Punicalagin A and B (and their derivatives), ellagic acid (and their derivatives) and gallic acid Total phenolic and flavonoid content Condensed and hydrolyzable tannins	(Çam and Hışıl, 2010)
Pomegranate seeds (*Punica granatum*)	Polyphenols: Caffeic acid derivative, catechin and kaempferol 3-O-rutinoside (among others) Maillard reaction products (browning and hydroxymethylfurfural) and reducing sugar and free amino acid content	(He et al., 2012)
Potato peels	Gallic acid, chlorogenic acid, caffeic acid, protocatechuic acid, syringic acid, p-hydroxyl benzoic acid, ferulic acid, and coumaric acid	(Singh and Saldaña, 2011)
White grapes (Viura variety)	Gallic acid, *p*-hidroxibenzoic acid, aesculetin, ferulic acid, scopoletin, sinapic acid and veratric aldehyde. caftaric acid, *cis* and *trans*-coutaric acids	(Palma et al., 2002)
Marine sources		
Microalgae (Chlorella vulgaris) and algae (Sargassum	Total phenolic compounds Carbohydrates Proteins Maillard reaction products (melanoidins and advanced glycation	(Plaza et al., 2010a)

Source	Principal compounds	Reference
vulgare, Porphyra spp., Cystoseira abies-marina, Sargassum muticum, Undaria pinnatifida, and Halopitys incurvus)	end products)	
Microalgae (*Haematococcus pluvialis*)	Phenolic compounds	(Rodriguez-Meizoso et al., 2010)

1.3.4.1 PHWE of essential oils

Several studies confirmed that many leafy spices and medicinal herbs, especially those belonging to the *Lamiaceae* family such as sage, rosemary, oregano and thyme, show strong antioxidant activity (Kahkonen et al., 1999; Ong et al., 2006). The main bioactive compounds of medicinal herbs may be divided in: i) essential oils, which contain a mixture of oxygenated compounds such as phenolic terpenes and hydrocarbons, and ii) non-volatile phenolic compounds such as flavonoids and phenolic acids (Ibañez et al., 2002; Zheng and Wang, 2001). In general, PHWE present several advantages over traditional extraction techniques for obtaining essential oils: low extraction times, higher quality of the extracts (mostly for essential oils), lower costs of the extracting agent, and environmentally friendly (Basile et al., 1998; Fernandez-Perez et al., 2000; Gámiz-Gracia and Luque de Castro, 2000; Herrero et al., 2006a; Kubátová et al., 2001; Soto Ayala and Luque de Castro, 2001). Basile et al. (1998) studied the principles involved in the PHWE process of essential oil from *Rosmarinus officinalis* (rosemary). The most water soluble compounds were removed rapidly and high temperatures increased the extraction rate. The extraction yields and costs were

compared with those obtained from a typical process of steam distillation (SD). The comparison between the extract obtained at 150 °C by PHWE with that obtained by SD showed that the PHWE is an effective methodology for the recovery of aromatic compounds from herbs. Particularly, a higher amount of oxygenated compounds was obtained by PHWE (with similar energy and water costs). However, the extraction of monoterpenes, hydrocarbons and large lipids, was less efficient. Similar results were found by Kubátová et al. (2001) in the PHWE of essential oils from savory and peppermint.

1.3.4.2 PHWE of flavonoids and phenolic acids

Flavonoids and phenolic acids have been mostly extracted using of organic solvents. Currently, the trend is to avoid organic solvents in the extraction process. PHWE present several features that make it an attractive alternative to traditional organic solvent extraction for the recovery of polyphenols from several plant materials: low cost, fast, temperature-dependent selectivity, human and environmental safe and efficient (Budrat and Shotipruk, 2009; Cheigh et al., 2012; Ju and Howard, 2005; Ko et al., 2011; Kumar et al., 2011; Rangsriwong et al., 2009). Budrat and Shotipruk (2009) compare PHWE with traditional solvent extraction methods (methanol extraction and Soxhlet water extraction) for the recovery of phenolic compounds from bitter melon. The antioxidant activity, the total phenolic content and the amount of individual phenolic compounds (e.g. catechin, genistic acid, gallic acid and chlorogenic acid) increased when increasing extraction temperature. Particularly, the total phenolic compounds and individual phenolics in the extracts obtained by PHWE, especially at 200 °C, were significantly higher than those in the extracts obtained by methanol extraction and Soxhlet water extraction. Moreover, the antioxidant activity of PHWE extracts was three times higher than the other extracts. Kumar et al. (2011) also reported pressurized hot water extracts of seabuckthorn with higher antioxidant activity and

polyphenolic content (quercetin-3-galactoside, kaempferol and isorhamnetin) than those obtained by Soxhlet and maceration methods. Similar results were found by Cheigh et al. (2012) for the extraction of flavanones form citrus peel (an agricultural by-product) when compared with conventional extraction methods using ethanol and methanol. In another work, Ju and Howard (2005), compared the PHWE of anthocyanins and other phenolics from grape skin (winery by-product) with conventional hot water or aqueous 60% (v/v) methanol extractions. The polyphenol content (anthocyanins, flavonols, hydroxycinnamates and phenolic acids) and the antioxidant activity of the extracts obtained using pressurized hot water (between 110 and 160 °C) were similar or slightly greater than those of the extracts obtained by conventional methods. However, the extraction time for the PHWE process was ten times shorter.

1.3.4.3 PHWE of condensed tannins

Condensed tannins, also known as proanthocyanidins, comprise a group of polyhydroxyflavan-3-ol oligomers and polymers of flavanols that can be linked to another polyphenol such as phenolic acids and anthocyanins (Haslam, 2007; Negro et al., 2003; Schofield et al., 2001). They can be found in high concentrations in grape seeds, plums, blackberries, cranberries and choke berries among fruits; sorghums, pinto beans and red kidney beans among cereals; hazelnuts and pecans among nuts and ground cinnamon among spices. They are also present in beverages and snacks, such as red wine, grape juice, baking chocolate and black chocolate, where they impart astringency and flavor (Gu et al., 2004; Xie and Dixon, 2005).

García-Marino et al. (2006) studied the PHWE of proanthocyanidins from grape seeds and compared the results with a methanol/water extraction at atmospheric pressure. PHWE showed good extraction yield, in some cases better than the traditional hydroalcoholic extraction. Also, it was reported that even higher recoveries can be obtained with sequential extraction at 50, 100 and 150 °C. Monrad et al. (2010) found

similar results when comparing PHWE at 140 °C with a conventional extraction (using acetone/water/acetic acid) in the recovery of proanthocyanidin monomers and oligomers from grape pomace. In liquid red grape extracts, proanthocyanidins can bind to anthocyanins to form polymeric pigments (Harbertson et al., 2003), another form of condensed tannins. It has been reported that the amount of polymeric pigments increases with temperature, especially above 100 °C (Ju and Howard, 2003). In that work, Ju and Howard compared the PHWE (acidified) of phenolics from grape skin with the traditional Soxhlet extraction. The results showed that the extraction (or formation) yield of polymeric pigments for PHWE was higher than that of the Soxhlet extraction with water (a similar yield was obtained when a mixture of water-methanol was used in the Soxhlet extraction).

1.4 PHWE of Polyphenols from Agro-industrial By-products (Deodorized Thyme and Grape Pomace): Bioactivity Assessment

In this section, the hypotheses that support this thesis work, as well as the general objectives that delimit the scope of this investigation are presented. In addition, a short description of the methodology used is described. Finally, the main results obtained, conclusions and prospects for future work are presented briefly.

Hypothesis

In the PHWE of polyphenols from plants materials the processing conditions, temperature and time, determine the polyphenols extraction yield, the formation of potentially harmful compounds, the antioxidant capacity and content of the extract, and, therefore, its bioactivity.

General objectives

1. To determine the effects of temperature and time on the extraction yield of polyphenols and total antioxidants from two agro-industrial by-products: deodorized thyme and grape pomace.

2. To characterize two pressurized hot water extracts obtained at different temperatures in terms of composition and chemical and biological antioxidant activity.

Results

The objective 1 was achieved in two stages:

a. *Determination of the effect of temperature and time on polyphenolic content and antioxidant activity in the pressurized hot water extraction of deodorized thyme (Thymus Vulgaris).* This work resulted in a publication accepted in the Journal of Agricultural and Food Chemistry and presented in the chapter 2 of this thesis.

   In this stage, the effects of temperature (50 to 200°C) and contact time (5 to 30 min) on the pressurized hot water extraction of deodorised thyme were explored for antioxidant activity, polyphenol profiles and total antioxidants (methodology details are presented in section 2.2). An inverse correlation was found between the antioxidant activity and total antioxidants with the amount and diversity of polyphenols. The highest total extract yield and antioxidant activity was obtained at 200 °C, although maximum polyphenol extraction yields of hydroxycinnamic acids, flavones, flavonols/flavanones and total polyphenols were detected at 100 ºC and 5 min. Higher temperatures and longer exposure times reduced extract polyphenol diversity. Dihydroxyphenyllactic acid was the only phenolic compound whose extraction yield increased with temperature, probably as a product of the thermal degradation of rosmarinic acid. Consequently, for extracting phenolics from thyme

100 ºC and 5 min would be appropriate operating conditions, whereas antioxidant-active non-phenolic compounds were favoured at higher temperatures and exposure times. Additionally, six not previously reported polyphenolic compounds were identified in thyme.

b. *Determination of the effect of pressurized hot water extraction on antioxidants from grape pomace before and after oenological fermentation*. This work resulted in a publication accepted in the Journal of Agricultural and Food Chemistry and presented in the chapter 3 of this thesis.

In this stage, grape pomace were extracted with pressurized hot water before and after fermentation, to explore the effects of extraction temperature (50-200°C) and time (5 and 30 min) on total extracted antioxidant levels and activity, and to determine the content and recovery efficiency of main grape polyphenols, anthocyanins, and tannins (methodology details are presented in section 3.2). Total antioxidant recovery and activity were not directly related to the main polyphenol content when performing pressurized hot water grape extraction of grape pomace. Elevating the extraction temperature increased total antioxidant extraction and antioxidant activity. However, maximum anthocyanin extraction yields were achieved at 100°C (5 min) and at 150°C (5 min) for tannins and tannin-anthocyanin adducts. Using higher temperatures and longer extraction times resulted in a sharp decrease of the extraction yield of these polyphenols, whereas the extraction of total antioxidant compounds was favoured at higher temperatures and exposure times.

The objective 2 was achieved in one stage:

a. *Characterization of pressurized hot water extracts of grape pomace: chemical and biological antioxidant activity*. This work resulted in a publication which was submitted to the Food Chemistry journal and is presented in the chapter 4 of this thesis.

Pressurized hot water extracts obtained at different temperatures possess different compositions and antioxidant activities and, consequently, different bioactivities. We characterized two pressurized hot water extracts from grape pomace obtained at 100°C (GPE100) and 200°C (GPE200) in terms of antioxidant activity and composition, as well as its protective effect on cell growth and mitochondrial membrane potential ($\Delta\psi_m$) in a HL-60 cell culture under oxidative conditions (methodology details are presented in section 4.2). GPE100 extracts were richer in polyphenols and poorer in Maillard reaction products (MRPs) than GPE200. Actually, hydroxymethylfurfural was detected only in GPE200. Both extracts exhibited similar protective effects on cell growth; comparable to the effect of Trolox. Moreover, GPE100 strongly decreased the $\Delta\psi_m$ loss, reaching values even lower than those of the control culture. This protective effect may be related to its high polyphenols content. At the highest concentration assessed, both extracts showed strong cytotoxicity, especially GPE200. This cytotoxicity could be related to their MRPs content.

Conclusions and future research

Different extraction conditions result in different antioxidants recovery, antioxidants profile and bioactivity of the extracts. High polyphenols amount could be extracted at moderate extraction temperature (100 °C), whereas at high temperatures a high recovery of total antioxidant can be achieved due to the formation of new antioxidants (some of which are potentially harmful). The protective effect of the extracts on cell cultures under oxidative conditions appears to be related to their content of both polyphenolic and non-polyphenolic antioxidants, whereas the Maillard compounds content determines their cytotoxicity. Therefore, further research should be focus on: polyphenols solubility and degradation at high temperatures, formation of neo-antioxidants and their bioactivity, and further purification processes.

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2 EFFECTS OF TEMPERATURE AND TIME ON POLYPHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY IN THE PRESSURIZED HOT WATER EXTRACTION OF DEODORISED THYME (THYMUS VULGARIS).

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2.1 Introduction

In the pressurized hot water extraction (PHWE) of polyphenols from plants the relationship between antioxidant activity and the polyphenol content is disputed, however (Budrat and Shotipruk, 2009; Ollanketo et al., 2002). The different positions may be due to diverse phenomena, such as thermal degradation, selective extraction of polyphenols or the formation of neo-antioxidant compounds, which are highly dependent on temperature and extraction time (Plaza et al., 2010b; Rodriguez-Meizoso et al., 2006). Irrespective, depending on the PHWE conditions used, it is possible to obtain extracts with different compositions, chemical activities and, consequently, different bioactive properties. But in order to define suitable operating conditions for a given PHWE process it is necessary to predetermine the qualitative and quantitative effects of a wide spectrum of extraction conditions.

Despite the many works in the literature on PHWE of spices (Ibañez et al., 2002; Ollanketo et al., 2002; Rodriguez-Meizoso et al., 2006), there is no comprehensive study that analyzed the impact of extraction conditions on both antioxidant activity and content of a wide spectrum of polyphenols.

In this research, a widely consumed spice, thyme (*Thymus vulgaris* L.), was considered as a case study. Biofunctional properties of thyme (antimicrobial, antifungal and antioxidant) are generally attributed to its essential oil content (Jiang et al., 2012;

Ziani et al., 2011). Around 65 different polyphenolic compounds have been identified in the genus *Thymus* (Vila, 2002). Even after extraction of the essential oil from thyme, the remaining solid contains polyphenolic compounds offering biological activity (Dorman et al., 2003), ranging from nutritional (Rubió et al., 2012) to strong anticancerogenic properties (Cai et al., 2011; Vargo et al., 2006). Previous research works that applied water to extract polyphenols from thyme leaves (Fecka et al., 2007; Kulisic et al., 2006) and deodorized thyme leaves (Dorman et al., 2003) limited maximum extraction temperature to 100°C.

In this work we aim to unmask the underlying relationship between antioxidant capacity and the polyphenolic composition of deodorized ground *Thymus vulgaris* extracts obtained under several PHWE conditions.

2.2 Materials and Methods

2.2.1 Chemicals

All organic solvents were HPLC gradient grade. Methanol and formic acid was purchased from Merck (Germany), acetonitrile was obtained from Scharlab (Spain). Reagents and standards used were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reactive and sodium carbonate were purchased from Merck. Tripyridyl Triazine (TPTZ), FeCl3(6H2O), fluorescein (FL,), pyrogallol red (PGR), 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, gallic acid, caffeic acid and quercetin were obtained from Sigma (USA). Apigenin was purchased from Extrasynthese (France).

2.2.2 Thyme origin and hydrodistillation treatment

Thyme (*Thymus vulgaris* L.) was obtained from an organic farm in X Region, Chile. A thyme herb sample (1 kg) was submitted to a process of steam distillation with distilled water for 3 h using a modified essential oil extractor (Steam Distillation Essential Oil Extractor, Figmay S.R.L.). Deodorized thyme from the hydrodistillation was separated into leaves and branches. The leaves were dried to reach equilibrium humidity (10% w/w) in a drying cabinet with forced ventilation at ambient temperature for 2 days. The dried leaves were ground in a four-knife mill to a particle size below 355 μm. The powder was stored at -4 °C until extraction.

2.2.3 PHWE

A sample of 5.00 g of dry leaf powder was mixed with neutral quartz sand to disperse the powder, reduce the volume of water used for the extraction and avoid filter clogging in the extraction cell. The mixture was then placed in a 100 mL stainless steel extraction cell. The plant material was extracted with about 100 mL of milli Q water in an Accelerated Extraction Equipment (ASE 150, Dionex). We performed a full factorial design in triplicate at 1500 psi with two factors, temperature and time, with four and three levels respectively. The operating conditions assessed were extraction temperature (50, 100, 150, and 200°C) and extraction time (5, 15, 30 min). After extraction, the cell content was rinsed with 100 mL of milli Q water and purged for 360 s applying pressurized nitrogen (150 psi). Finally, the extracts were freeze-dried and stored in amber vials at -20°C until analysis. A 1 g/L of dried extract solution was prepared for analysis.

2.2.4 Determination of DPPH radical scavenging activity

Extract anti-radical capacity was determined using the DPPH radical-scavenging method (Brand-Williams et al., 1995). Initially, a stock solution was prepared by dissolving 23.5 mg of DPPH reagent in 100 mL of methanol. The DPPH solution was prepared by diluting (1:10 v/v) the stock solution with methanol. A volume of 50 µL of extract solutions at different concentrations was mixed with 2 mL of DPPH solution. The bleaching of DPPH was measured at 516 nm (DR 2000 Spectrophotometer, Hach) until the absorbance remained unchanged (30 min approx.) in the dark and at room temperature. The effective concentration of thyme extract to reach a 50% of inhibition of DPPH radical absorption, IC50 (mg/L), was calculated. Then, the antioxidant capacity was compared with Trolox, a synthetic hydrophilic vitamin E analogue, using the Trolox equivalent antioxidant capacity (TEAC) equation: TEAC = IC50 Trolox/IC50 sample (Clariana et al., 2011). DPPH values were expressed as mg of Trolox equivalent (TE) per grams of dry plant (d.p.).

2.2.5 Determination of ferric reducing antioxidant power (FRAP)

The FRAP test offers a putative index of antioxidant or reducing capacity of antioxidants in the sample (Benzie and Strain, 1996). A working solution was prepared by mixing; 300 mM of acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl and a freshly prepared 20 mM $FeCl_3(6H_2O)$ solution in 10:1:1 (v/v) proportion. For the assay 3 mL of working reagent was mixed with 100 µL of sample or calibration standard. Absorbance was measured at 593 nm (DR 2000 Spectrophotometer, Hach) after a resting period of 30 min (Pulido et al., 2000). A calibration curve was constructed using ascorbic acid (0.1-0.8 mM). The regression coefficient of ascorbic acid was 0.9989. The results were expressed as ascorbic acid equivalent (AAE) per g of d.p.

2.2.6 Determination of oxygen radical absorbance capacity (ORAC)

Consumption of the probe molecule, FL or PGR, associated with its incubation in the presence of a peroxyl radical source, AAPH, was estimated from fluorescence (F) and absorbance (A) measurements, respectively (López-Alarcón et al., 2007). Values of (F_i/F_0) or (A_i/A_0) were plotted as a function of time where F_0 and A_0 are the initial fluorescence and absorbance, respectively, and F_i and A_i are the fluorescence and absorbance reading at time i, respectively. Integration of the area under the curve (AUC) was performed up to a time such that (F_i/F_0) or (A_i/A_0) reached a value of 0.2. Stock solutions of PGR (1 x 10^{-4} M) or FL (1 x 10^{-5} M) were prepared daily in phosphate buffer 75 mM, pH 7.4. A reaction mixture containing AAPH (10 mM), PGR (5 mM), with or without the tested sample, in phosphate buffer, was incubated at 37 ºC in the thermo-cuvette of a UV-visible spectrophotometer (Hewlett Packard 8453). PGR consumption was evaluated from the progressive absorbance decrement measured at 540 nm. A similar procedure was carried out employing FL (70 nM), but in this case, consumption was assessed from the decrease in sample fluorescence intensity (excitation: 493 nm; emission 515 nm) in a spectrofluorimeter (Aminco-Bowman Series 2). A phosphate buffer was used as a blank, and different Trolox concentrations (12.5, 25, 50, and 100 µM) were used to prepare a standard curve.

The TEAC value was calculated as

$$TEAC = \left[\frac{(AUC_{Sample} - AUC_{Blank})}{(AUC_{Trolox} - AUC_{Blank})}\right] \times \left(\frac{conc.\,of\ Trolox}{conc.\,of\ sample}\right)$$

Results were expressed as TE per g of d.p.

2.2.7 Determination of total polyphenols (TA) by Folin assay

Given that Folin values systematically exceed the total polyphenol content values, the results obtained using the Folin-Ciocalteu assay (Singleton and Rossi, 1965) were considered in this work as total antioxidant values (Perez-Jimenez et al., 2010). A volume of 4.25 mL of phenolic extract (1 mg/mL) and 0.25 mL of Folin-Ciocalteu reactive were diluted with distilled water (1/1 v/v) and 0.5 mL of a sodium carbonate solution (10% w/v). Absorbance was measured at 765nm (DR 2000 Spectrophotometer, Hach) after a reaction time of 1 h at room temperature. A calibration curve was constructed using gallic acid as the calibration standard (20-90 mg/L). The regression coefficient of gallic acid was 0.9987. Results were expressed as gallic acid equivalent (GAE) per g of d.p.

2.2.8 Qualitative and quantitative polyphenol analysis by HPLC-ESI-Q-TOF mass spectrometry

HPLC-ESI-Q-TOF equipment was used to identify compounds by MS/MS analysis and for the relative quantification of polyphenol subclasses. The ESI-Q-TOF instrument was a Quadrupole time-of-flight QStar Elite (AB Sciex) combined with high-performance liquid chromatography Agilent 1200 RRLC system (Agilent, Waldbronn, Germany). Separations were conducted using a Luna® C18 3.5 µm particle size column (50 x 2.1mm i.d.; Phenomenex, Torrance, CA, USA). Solutions for the mobile phase were: 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The following gradient was applied at a flow rate of 0.40 mL/min: 0 min, 8% B; 10 min, 23% B; 15 min, 50% B; 20 min, 50% B; 30 min, 70% B; 31 min, 100% B; 35 min, 100% B and finally, 36 min, 8% B. The final condition was held for 9 min as a re-equilibration step. The injection volume was 25 µL. For MS analysis, data acquisition was performed using negative ion mode the mass range was from 70 to 600 Da. Data

were acquired using a declustering potential of -180V, focusing potential of -190V, declustering potential two of -10V, and source temperature of 400 ºC. TOF mode was used to determine relative quantification of phenolic compounds and MS/MS experiments were performed in the IDA (information-dependent acquisition) mode, with a collision energy of 35 V. Analyst 2.0 software from AB Sciex was used for data acquisition and processing. Peak identity was established by comparing the molecular formula proposed by the software for the different peaks obtained in the MS experiments with those included in the Phenol-Explorer database (Neveu et al., 2010), as well as with specific papers on thyme polyphenols. These identities were confirmed from the MS/MS experiments, comparing the main fragments with those previously described for the same compound (Hossain et al., 2010; Parejo et al., 2004) and using the general fragmentation patterns reported for polyphenols (Fabre et al., 2001). Compounds identified by LC-MS/MS analysis were grouped into 4 subclasses. These were quantified relatively using a corresponding standard: gallic acid (structurally related to hydroxyphenylpropanoic acids) for hydroxyphenylpropanoic acids ($y = 5244.1x$, $R^2 = 0.9781$, LOD = 20.9 µg/L, LOQ = 69.5 µg/L); caffeic acid for hydroxycinnamic acids ($y = 10026x$, $R^2 = 0.9834$, LOD = 2.2 µg/L, LOQ = 7.4 µg/L); apigenin for flavones ($y = 11093x$, $R^2 = 0.9795$, LOD = 4.2 µg/L, LOQ = 14.1 µg/L); quercetin for flavonols and flavanones ($y = 6214x$, $R^2 = 0.9560$, LOD = 3.7 µg/L, LOQ = 12.5 µg/L). The extraction yield of each polyphenol subclass was expressed as mg of standard per g of d.p. The values of the different subclasses at each extraction condition were added to obtain the total polyphenol extraction yield.

2.2.9 Statistical analysis

Extraction and analysis were performed in triplicate with the data presented as mean ± SD values. StatgraphicsPlus for Windows 4.0 (Herndon, VA) was used for statistical analyses. In order to study the effects of the temperature and extraction time

on extraction performance as well as the interaction between these two factors, analysis of variance (Factorial ANOVA) and least significant difference (LSD) tests were applied on the response variables with a significance of $p \leq 0.05$.

2.3 Results and Discussion

To have a common base for comparison, results of all the analyses of the extracts are expressed in terms of mass of dried plant (d.p.) before extraction.

2.3.1 Effect of temperature and time on total extract yield

Powdered thyme leaves were extracted at four temperatures, 50, 100, 150 and 200°C, and at three extraction times, 5, 15 and 30 min. The dry extracts obtained were weighed and the total extract yield (TEY) was expressed as the percentage of extract recovered per d.p. processed (Table 2.1).

Extraction temperature had a statistically significant effect (p-value < 0.0001) on TEY. Even though yields obtained at 50 and 100ºC were similar. Increasing the extraction temperature from 100 to 200°C resulted in a more than twofold increase in TEY. This effect of temperature confirms previous observations (Herrero et al., 2010; Plaza et al., 2010a) in which two main phenomena are involved; an increase in solubility and hydrolytic reactions. The use of higher temperatures increases the capacity of water to solubilise analytes (Ong et al., 2006). At temperatures of 160°C and higher, pressurized hot water is even able to solubilise hemicellulose (Ingram et al., 2009) and lignin (Liu and Wyman, 2003). Moreover, during a water-based thermal process, part of the hemicellulose is hydrolyzed and forms acids. These acids are assumed to catalyze hydrolysis of remaining hemicelluloses (Gregg and Saddler, 1996). Effects of these reactions can be observed clearly at 150°C where the extraction yield increased over

time. Hydrolysis of lignocellulosic material in PHWE of polyphenol-rich materials, which is usually overlooked, may contribute to the release of phenolic cell wall-associated compounds (Pérez-Jiménez and Torres, 2011). It may also release other compounds, such as reducing sugars, which may alter the Folin assay measurement (Kumazawa et al., 2001).

Extraction time had no statistically significant effect on total extraction yield. In addition, no correlation on the extraction yield was detected in the interaction between temperature and time factors. Little to no influence of PHWE time on extraction yield has been reported previously for a similar biomass (Herrero et al., 2006b).

Table 2.1 Extraction yield and antioxidant activities obtained at the different extraction conditions of *thymus vulgaris* polyphenols with pressurized hot water.

Temp. (°C)	Time (min)	Extraction yield (%)	TA (mg GAE/g d.p.)	DPPH (mg TE/g d.p.)	FRAP (mg AAE/g d.p.)	ORAC-FL (mg TE./g d.p.)	ORAC-PGR (mg TE./g d.p.)
50	5	33.6 ± 0.8^{a}	131 ± 15^{a}	14.2 ± 2.0^{a}	96 ± 10^{a}	61.7 ± 1.5^{cd}	40.9 ± 2.2^{ab}
	15	35.5 ± 3.8^{a}	159 ± 18^{ab}	14.0 ± 1.2^{a}	112 ± 8^{abc}	49.3 ± 0.4^{ab}	43.5 ± 2.4^{b}
	30	34.7 ± 1.6^{a}	150 ± 7^{ab}	13.8 ± 0.2^{a}	107 ± 11^{ab}	42.9 ± 1.7^{ab}	39.2 ± 3.7^{ab}
100	5	37.4 ± 4.2^{a}	168 ± 12^{b}	15.2 ± 0.3^{abc}	118 ± 2^{abc}	45.3 ± 2.7^{ab}	38.4 ± 4.3^{ab}
	15	39.1 ± 2.1^{a}	168 ± 7^{b}	14.9 ± 0.7^{abc}	123 ± 14^{bc}	48.5 ± 4.4^{ab}	34.3 ± 3.7^{ab}
	30	38.2 ± 4.8^{a}	160 ± 13^{ab}	14.7 ± 0.6^{ab}	111 ± 4^{abc}	41.1 ± 1.4^{a}	32.5 ± 1.2^{a}
150	5	48.9 ± 0.7^{b}	199 ± 3^{c}	17.1 ± 1.6^{bcd}	131 ± 12^{bcd}	53.5 ± 4.9^{bc}	56.6 ± 2.4^{c}
	15	54.8 ± 0.8^{bc}	202 ± 3^{c}	17.7 ± 0.2^{cd}	132 ± 10^{cd}	61.1 ± 0.6^{cd}	54.4 ± 2.0^{c}
	30	56.7 ± 1.6^{c}	221 ± 2^{cd}	18.1 ± 0.3^{d}	156 ± 14^{e}	63.9 ± 2.9^{cd}	62.0 ± 5.7^{c}
200	5	59.0 ± 1.7^{c}	260 ± 0^{e}	21.4 ± 1.4^{e}	152 ± 3^{de}	69.7 ± 6.4^{d}	64.8 ± 3.9^{cd}
	15	60.1 ± 1.0^{c}	262 ± 6^{e}	19.0 ± 0.9^{de}	166 ± 8^{e}	68.7 ± 2.0^{d}	72.5 ± 5.5^{d}
	30	55.8 ± 2.3^{c}	247 ± 22^{de}	19.9 ± 1.3^{de}	155 ± 6^{e}	89.6 ± 9.4^{e}	73.1 ± 1.5^{d}

2.3.2 Effect of temperature and time on total antioxidants and antioxidant activity

Extraction temperature was the only factor that had a statistically significant effect (all p-values < 0.0001) on TA and antioxidant activity tests. The results are listed in Table 2.1. TA values increased with temperature. A temperature increment from 50 to 200°C resulted in a twofold increase in TA from 131 to 260 mg GAE/g d.p. This temperature effect on TA has been well reported in PHWE of other plants (Herrero et al., 2010; Hossain et al., 2011). In addition, an increase in extraction temperature from 50 to 200°C resulted in an increase of DPPH radical scavenging and ferric ion reducing activities of more than 50%. These results are in agreement with the findings of similar research where the temperature was found to be the dominant factor to attain high antioxidant activity (Hossain et al., 2011). The effect of temperature on ORAC-FL and ORAC-PGR assays (scavenging of peroxyl radicals) were the same as those on DPPH and FRAP assays (reducing activity). Therefore, the antioxidants extracted were equally efficient under different experimental set-ups and both the amount of antioxidants and the their reactivity increased with temperature (López-Alarcón et al., 2007).

Correlation coefficients between TA and antioxidant activity obtained from the different assays are shown in Table 2.2. Despite these assays being based on different chemical mechanisms, a strong correlation was observed between them all; corroborating that temperature has the same effect on TA as it has on antioxidant activity. This observation agrees with previous findings (Budrat and Shotipruk, 2009; Hassas-Roudsari et al., 2009).

Table 2.2 Pearson' correlation coefficients between antioxidant activity assays and total antioxidants

Correlated assays	Correl. Coef.
DPPH - FRAP	0.90
DPPH - ORAC(FL)	0.80
DPPH - ORAC(PGR)	0.90
FRAP - ORAC(FL)	0.72
FRAP - ORAC(PGR)	0.90
ORAC(FL) - ORAC(PGR)	0.87
TA - DPPH	0.96
TA - FRAP	0.97
TA - ORAC(FL)	0.75
TA - ORAC(PGR)	0.92

Under most conditions studied, TA and antioxidant activity values increased with increasing extraction temperature; the highest values for all assays were obtained at the maximum temperature (Table 2.1). The antioxidant activities obtained at 50 and 100ºC were similar and significantly increased above 100ºC. The increase in antioxidant activity and TA obtained at high temperatures might be due to the increase in TEY. However, apart from an enhancement in solubility and diffusion rate, several reactions may generate compounds with antioxidant activities at temperatures above 100ºC (Martins et al., 2000; Plaza et al., 2010a).

2.3.3 Polyphenol profile of extracts obtained under different extraction conditions

2.3.3.1 Compound identification

Mass spectral data for compounds identified in negative ionisation mode are listed in Table 2.3, where a summary of the proposed identification is presented. Seventeen phenolic compounds were identified from the extracts obtained, among which, six compounds have not been reported previously in the genus *Thymus*. 3,4-dihydroxyphenyllactic acid (DHPLA) was the only compound identified as hydroxyphenylpropanoic acid. This compound has not been reported previously in the genus *Thymus*, despite being one of the o-diphenol moieties constituting rosmarinic acid. Four compounds belonging to hydroxycinnamic acids were identified; caffeic and rosmarinic acids and two compounds not reported previously in genus *Thymus*: caffeic acid 4-*O*-glucoside and dihydrocaffeic acid. In the flavone subclass, four glycosilated compounds (apigenin 6,8-di-*C*-glucoside, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide and apigenin 7-*O*-glucuronide), one aglycone, (luteolin), and three methoxylated flavones, (cirsimaritin, cirsilineol and 5,6-dihydroxy-7,8,3',4'-tetramethoxyflavone, not previously reported in *Thymus*) were detected. Also identified were two glycosilated flavonols: (quercetin 3-*O*-glucoside and dihydrokaempferol 3-*O*-glucoside), for which only aglycone form had been previously reported in thyme. Finally, one glycosylated flavanone, eriocitrin, and one phenolic terpene, carnosol, were identified. Some of the newly identified compounds are glycosylated conjugates of already described aglycones. The more hydrophilic conjugates may have been concentrated in our starting material that is the remaining leaves from steam distillation of the hydrophobic essential oil. The distillation at 100ºC might have also concentrated the methoxylated flavones in the leaves. The new methoxylated flavones might have

been more efficiently extracted with pressurized hot water that shows a dielectric constant and polarity lower than steam.

Concerning the six new compounds found in thyme, it is worth pointing out that knowledge of the polyphenol composition of many plants is often limited to one or a few varieties. Furthermore, numerous other factors may affect the polyphenol content of plants; ripeness at the time of harvest, environmental factors, processing (pretreatment, extraction method and drying) and storage (Manach et al., 2004). In our study, a comprehensive revision of the results of LC-MS/MS analysis was conducted in an attempt to identify a wide spectrum of polyphenols and not just specific given compounds.

Identification of these compounds was confirmed by comparison between observed and calculated mass (errors below 10 ppm) and by the fragmentation pattern obtained when performing MS/MS analysis (Table 2.3). For instance, for phenolic compounds found in thyme, either glucosilated or glucuronidated, aglycone fragments (e.g., fragment at *m/z* 285 for both luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide) were detected and in the case of phenolic acids (e.g., caffeic acid or dihydrocaffeic acid) losses of CO_2 were observed upon fragmentation.

Table 2.3 Polyphenols found by HPLC-ESI-Q-TOF in *thymus vulgaris* extracts obtained with pressurized hot water.

Gluc : glucoside; A: aglycone; Glur: glucoronide; DHPLA: 3,4-dihydroxyphenyllactic acid a: typical of di-C-glycosylflavonoids; b: associated with a loss of a disaccharide. Data from the extract obtained at 100°C and 5 min was used to calculate the error value (ppm).

Compound	Rt (min)	$[M-H]^-$ exp	$[M-H]^-$ calc	ppm	Characteristic MS/MS ion fragments m/z		Reported in genus *Thymus*
3,4-Dihydroxyphenyllactic acid	1.4	197.0432	197.0450	-9.1	135	$[M-H-H_2O-CO_2]$	No
					123	$[M-H-C_3H_6O_2]$	
Caffeic acid 4-O-glucoside	2.9	341.0862	341.0873	-3.1	179	[M-H-Gluc]	No (aglycone only)
					135	$[M-H-Gluc-CO_2]$	

Caffeic acid	4.1	179.0342	179.0344	-1.3	135	[M-H-CO_2]	
Dihydrocaffeic acid	4.9	181.0503	181.0500	1.2	137	[M-H-CO_2]	No
Apigenin 6,8-di-C-glucoside	5.4	593.1504	593.1506	-0.4	503	[M-H-90][a]	(Vila, 2002)
					473	[M-H-120][a]	
					383	[A+113][a]	
					353	[A+83][a]	
Quercetin 3-O-glucoside	7.5	463.0914	463.0877	8.1	301	[M-H-Gluc]	No (aglycone only)
Eriocitrin	8.4	595.1707	595.1663	7.4	287	[M-H-308][b]	(Fecka and Turek, 2008)
					147	-	
Dihydrokaempferol 3-O-glucoside	8.7	449.1085	449.1084	0.3	287	[M-H-Gluc]	No (aglycone only)
					151	-	
					135	-	
Luteolin 7-O-glucoside	9.6	447.0896	447.0927	-7.0	285	[M-H-Gluc]	(Hossain et al., 2010)
Luteolin 7-O-glucuronide	10.2	461.0737	461.0720	3.7	285	[M-H-Glur]	(Dapkevicius et al., 2002)
Apigenin 7-O-glucuronide	12.6	445.0753	445.0771	-4.0	269	[M-H-Glur]	(Fecka and Turek, 2008)
Rosmarinic acid	12.8	359.0785	359.0767	5.0	197	[DHPLA-H]	(Hossain et al., 2010)
					179	[Caffeic acid-H]	
					135	[Caffeic acid-H-CO_2]	
Luteolin	16.5	285.0394	285.0399	-1.8	199	[M - H - CH_2O_2-CO_2]	(Fecka and Turek, 2008)
					175	[M-H-C_3O_2-CH_2O_2]	
					151	Lactone forme A^- ring	
					133	B^- ring	
Cirsimaritin	19.2	313.0703	313.0712	-2.9	298	[M-H-CH_3]	(Vila, 2002)
					283	[M-H- CH_3-CH_3]	
Cirsilineol	19.5	343.0812	343.0817	-1.7	328	[M-H-CH_3]	(Vila, 2002)
					313	[M-H-CH_3-CH_3]	
					298	[M-H- CH_3-CH_3-CH_3]	

5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone	20.1	373.0899	373.0923	-6.5	343	[M-H-CH_3-CH_3]	No
					328	[M-H-CH_3-CH_3-CH_3]	
Carnosol	20.9	329.1766	329.1752	4.0	285	[M-H-CO_2]	(Hossain et al., 2010)

2.3.3.2 Effect of extraction temperature and time on qualitative polyphenol profile

Different extraction conditions tested caused differences in the phenolic profiles of the extracts (Table 2.4). Some of the phenolic compounds (apigenin 7-*O*-glucoside, luteolin, cirsimaritin, cirsilineol and 5,6'-dihydroxy-7,8,3',4'-tetramethoxyflavone) were not detected at 200 ºC for any extraction time while other compounds (caffeic acid 4-*O*-glucoside, quercetin 3-*O*-glucoside and eriocitrin) were detected only at shorter extraction times. Dihydroxykaempferol 3-*O*-glucoside was especially labile; hence, it was only detected in the 5 min extractions at 100ºC and at 150ºC. Dihydrocaffeic acid, in turn, was detected only at 200°C and 30 min and it is possible that the appearance of this compound is due to the loss of unsaturation (the double bond) in the caffeic acid chain from its exposure over a long time at high temperature. Finally, luteolin 7-*O*-glucuronide was not detected at 50°C and 5 min extraction, which would appear to indicate that the conditions were not sufficiently conducive.

In summary, high extraction temperatures and in some cases long extraction times, produce extracts with a small number of polyphenols, notably methylflavones and flavonols.

Table 2.4 Polyphenols identified in extracts obtained at different extraction conditions of temperature and time, analyzed by HPLC-ESI-Q-TOF.
Symbol x indicates presence of the compound in the corresponding extraction condition.

Polyphenol Subclass	Compound	Extraction conditions												
		50			100			150			200			(°C)
		5	15	30	5	15	30	5	15	30	5	15	30	(min)
HPPA	DHPLA	X	X	X	X	X	X	X	X	X	X	X	X	
Hydroxycinnamic Acids	Caffeic acid 4-*O*-glucoside	X	X	X	X	X	X	X	X	X	X	X		
	Caffeic acid	X	X	X	X	X	X	X	X	X	X	X	X	
	Dihydrocaffeic acid												X	
	Rosmarinic acid	X	X	X	X	X	X	X	X	X	X	X	X	
Flavones	Apigenin 6,8-di-*C*-glucoside	X	X	X	X	X	X	X	X	X	X	X	X	
	Luteolin 7-*O*-glucoside	X	X	X	X	X	X	X	X	X	X	X	X	
	Luteolin 7-*O*-glucuronide		X	X	X	X	X	X	X	X	X	X	X	
	Apigenin 7-*O*-glucuronide	X	X	X	X	X	X	X	X	X				
	Luteolin	X	X	X	X	X	X	X	X	X	X	X	X	
	Cirsimaritin	X	X	X	X	X	X	X	X	X				
	Cirsilineol	X	X	X	X	X	X	X	X	X				
	DHTMF	X	X	X	X	X	X	X	X	X				
Flavonols	Quercetin 3-*O*-glucoside	X	X	X	X	X	X	X	X	X	X	X		
	DHKG	X	X	X	X			X						
Flavanones	Eriocitrin	X	X	X	X	X	X	X	X	X	X			
Phenolic terpenes	Carnosol	X	X	X	X	X	X	X	X	X	X	X	X	

HPPA: Hydroxyphenylpropanoic acid; DHPLA: 3,4-dihydroxyphenyllactic acid; DHTMF: 5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone; DHKG: Dihydrokaempferol 3-*O*-glucoside

2.3.4 Effect of temperature and time on the extraction of polyphenol subclasses

The effects of temperature and extraction time on the polyphenol extraction yields were assessed through the relative quantification of the different polyphenol subclasses (Figure 2.1; Table 2.5). Temperature, time (except for flavones) and the interaction of these factors had a statistically significant effect on extraction yield of all polyphenol subclasses analysed. Temperature showed p-values equal to 0.0000 for all subclasses and total polyphenols. Extraction time showed p-values <0.0001, 0.0040, <0.0001 and 0.0126 for flavonols, hydroxyphenylpropanoic acids, hydroxycinnamic acids and total polyphenols, respectively. The interaction of temperature and time factors showed p-values 0.0014, 0.0002, 0.0095, <0.0001 and 0.0021 for flavones, flavonols, hydroxyphenylpropanoic acids, hydroxycinnamic acids and total polyphenols, respectively.

The impact of temperature on the extraction yield of total polyphenols, hydroxycinnamic acids, flavones and flavonols/flavanones is similar (Figure 2.1). The lowest yields were obtained at 200°C and the highest at 100 °C. At 50 and 150°C the yields were also affected by exposure time. The extraction yields of flavones and total polyphenols at 50°C increased with time, while that of flavonols and hydroxycinnamic acids remained practically the same. In turn, at 150°C all yields were negatively affected by the exposure time, except for hydroxyphenylpropanoic acids. In fact, this subclass presents a completely different pattern (Figure 2.1). No statistical difference in yields obtained at 50 and 100°C for any exposure time were observed. In addition, at 150 and 200°C the extraction yield increased with exposure time.

In summary, the highest extraction yields were achieved at 100ºC and 5 min for hydroxycinnamic acids, flavones, flavonols/flavanones, and for total polyphenols (27.4 mg polyphenols/g d.p.). The extraction yield of hydroxyphenylpropanoic acids showed a maximum at 200°C and 15 min.

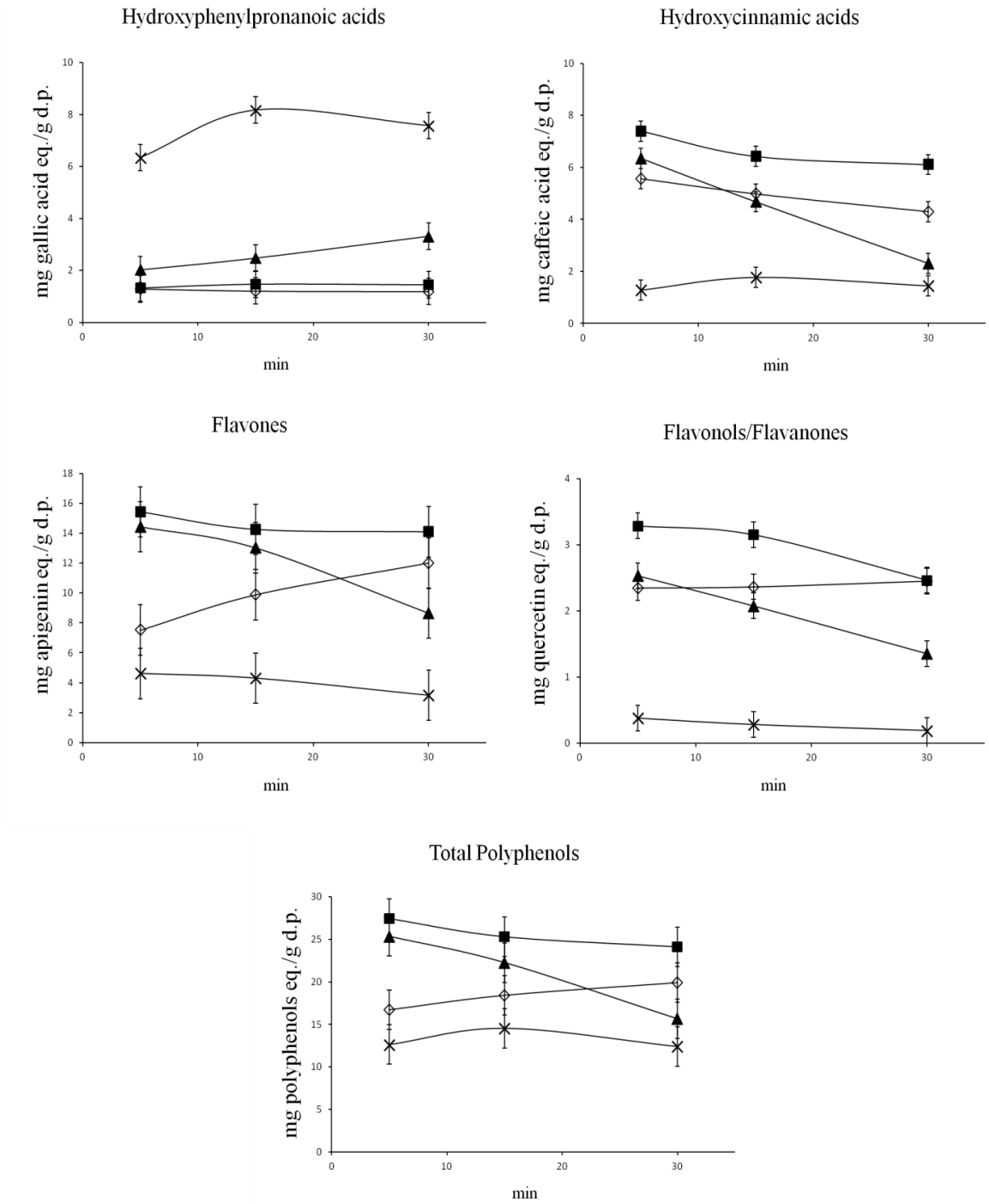

Figure 2.1 Extraction dynamics of polyphenol subclasses and total polyphenols.

Curve symbols, ◊, ■, ▲ and ×, correspond to the extraction temperatures 50, 100, 150 and 200°C, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

Table 2.5 Polyphenol extraction yield in *thymus vulgaris* obtained with pressurized hot water analyzed by HPLC-ESI-Q-TOF

Temp (°C)	Time (min)	HPPA (mg gallic acid eq/ g d.p.)	HCA (mg caffeic acid eq/ g d.p.)	Flavones (mg apigenin eq/ g d.p.)	Flavonols/Flavanones (mg quercetin eq/ g d.p.)	Polyphenols (mg polyphenol eq/ g d.p.)
50	5	1.29 ± 0.08^{a}	5.57 ± 0.06^{de}	7.53 ± 0.01^{bc}	2.35 ± 0.05^{cd}	16.74 ± 0.10^{abc}
	15	1.22 ± 0.06^{a}	4.98 ± 0.04^{cd}	9.88 ± 0.96^{cde}	2.37 ± 0.07^{cd}	18.44 ± 0.79^{bcd}
	30	1.20 ± 0.03^{a}	4.30 ± 0.32^{c}	11.99 ± 1.58^{def}	2.45 ± 0.00^{cd}	19.93 ± 1.92^{cde}
100	5	1.34 ± 0.00^{a}	7.39 ± 0.24^{g}	15.42 ± 1.04^{g}	3.29 ± 0.00^{e}	27.43 ± 1.28^{g}
	15	1.47 ± 0.15^{ab}	6.43 ± 0.22^{f}	14.25 ± 1.73^{fg}	3.15 ± 0.15^{e}	25.31 ± 1.52^{fg}
	30	1.46 ± 0.07^{a}	6.11 ± 0.17^{ef}	14.09 ± 0.84^{fg}	2.47 ± 0.15^{d}	24.11 ± 1.23^{efg}
150	5	2.04 ± 0.31^{ab}	6.34 ± 0.42^{f}	14.43 ± 1.23^{fg}	2.53 ± 0.15^{d}	25.34 ± 2.11^{fg}
	15	2.48 ± 0.07^{bc}	4.67 ± 0.41^{c}	13.01 ± 1.70^{efg}	2.08 ± 0.23^{c}	22.25 ± 2.41^{def}
	30	3.32 ± 0.37^{c}	2.31 ± 0.27^{b}	8.66 ± 1.22^{cd}	1.36 ± 0.24^{b}	15.65 ± 2.10^{abc}
200	5	6.35 ± 0.61^{d}	1.28 ± 0.05^{a}	4.61 ± 0.34^{ab}	0.38 ± 0.02^{a}	12.62 ± 0.88^{a}
	15	8.17 ± 0.63^{e}	1.77 ± 0.28^{ab}	4.30 ± 0.28^{ab}	0.28 ± 0.04^{a}	14.53 ± 1.23^{ab}
	30	7.57 ± 0.52^{e}	1.45 ± 0.10^{a}	3.17 ± 0.01^{a}	0.19 ± 0.06^{a}	12.39 ± 0.68^{a}

Values with the same superscript indicate no statistically significant difference between extraction conditions at the confidence interval of 95%. HPPA: hydroxyphenylpropanoic acids; HCA: hydroxycinnamic acids; d.p.: dry plant

Flavonoids are known to degrade in water at temperatures of 100°C and above (Palma et al., 2001; Srinivas et al., 2011). The number and type of substituents as well as the position of the hydroxyl group affect flavonoid thermal stability, as compounds possessing a smaller number of substituents are less stable at high temperatures (Magdalena, 2011). In the case of phenolic acids the effect of temperature is especially marked. For example, caffeic acid and DHPLA are the two hydrolyzed degradation products of rosmarinic acid (Nakamura et al., 1998). As seen in Figure 2.2, the

extraction yield of rosmarinic acid is lowest at 200°C and at 150°C is highly sensitive to the exposure time, suggesting degradation. On the other hand, the extraction yield of DHPLA at 150°C increased gradually with extraction time, while its extraction yield was highest at 200°C. To the best of our knowledge, there are no prior studies on the formation of DHPLA from polyphenolic degradation of rosmarinic acid at high temperatures. However, only a slight increase in the extraction yield of caffeic acid with temperature was found, reaching a maximum at 150°C. Hossain *et al*. (Hossain et al., 2011) also observed a slight decrease of extraction yield of rosmarinic acid and an increase of caffeic acid using pressurized hot methanol/water extraction at a temperature of around 150°C. In an attempt to explain this behaviour, some researchers have reported the formation of simple catechol monomers such as 4-vinylguaiacol and 4-vinylphenol from the thermal decarboxylation of caffeic acid in an aqueous system at temperatures between 90 and 100°C (Vanbeneden et al., 2008). Moreover, caffeic acid subjected to mild pyrolysis (225 °C - 226 °C) under vacuum, resulted in rapid decarboxylation and the formation of tetraoxygenated phenylinadan isomers, showing strong antioxidant activity (Guillot et al., 1996). Therefore, it seems that the caffeic acid formed after rosmarinic acid decomposition is rapidly degraded and transformed into other derived compounds.

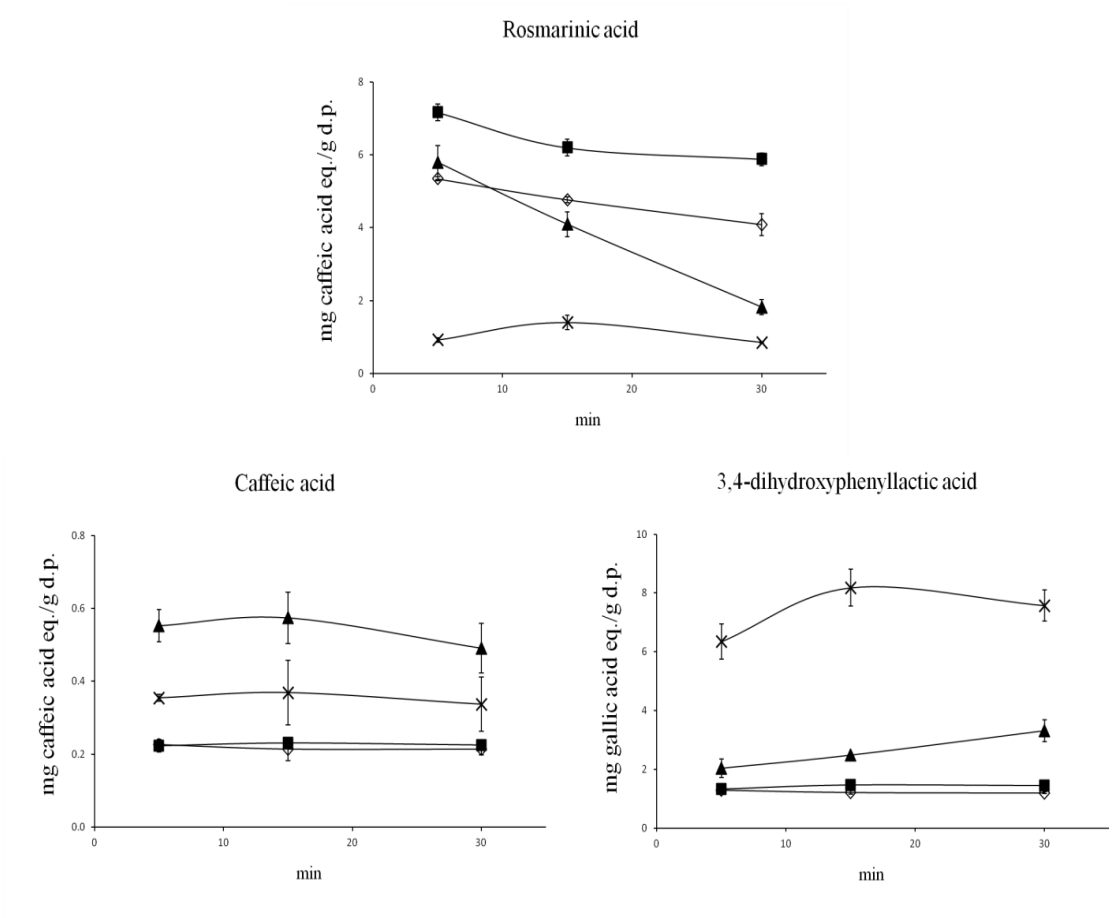

Figure 2.2 Extraction dynamics of rosmarinic acid, caffeic acid and 3,4-hydroxyphenillactic acid.

Curve symbols, ◊, ■, ▲ and ×, correspond to the extraction temperatures 50, 100, 150 and 200 °C, respectively. Bars represent the standard deviation of 3 replicates.

2.3.5 Correlation between polyphenol subclasses, total antioxidants and antioxidant activity

Quantification results obtained from LC-MS were correlated with both antioxidant and TA analysis using all of the values (corresponding to the 12 extraction conditions). The results obtained (Table 2.6) show a strong negative correlation between chemical antioxidant assays and hydroxycinnamic acids, flavones, flavonols/flavanones

and total polyphenols (R values ranging from -0.592 to -0.898), but a strong positive correlation with DHPLA (R values ranging from 0.838 to 0.918). However, the increase in the DHPLA does not in itself explain the increase in antioxidant activity since in chemical assays phenolic acids present similar antioxidant activities to flavonoids (Rice-Evans et al., 1996) and, even DHPLA has shown less antioxidant activity than rosmarinic acid (Song et al., 2010). This suggests that factors other than the amount of polyphenols in the extracts play a significant role in determining antioxidant activity and TA.

Table 2.6 Pearson' correlation coefficients between extraction yield values of polyphenol subclasses determined by HPLC-ESI-Q-TOF and antioxidant activity assays

Assays	HPPA	HCA	Flavones	Flavonols/ Flavanones	Total Polyphenols
TA	0.918	-0.809	-0.649	-0.866	-0.592
DPPH	0.866	-0.785	-0.649	-0.848	-0.592
FRAP	0.869	-0.779	-0.593	-0.809	-0.592
ORAC-FL	0.838	-0.790	-0.846	-0.856	-0.786
ORAC-PGR	0.893	-0.842	-0.759	-0.898	-0.716

HPPA: hydroxyphenylpropanoic acids; HCA: hydroxycinnamic acids

In aqueous systems at high temperatures many plant material reactions produce antioxidants or reducing compounds. The high antioxidant activity of extracts obtained at 150°C and above may be associated, in part, with the generation of antioxidant-capable Maillard reaction products such as melanoidins (Hossain et al., 2011). In addition, thermal decomposition products from polyphenols, such as caffeic acid, have revealed stronger antioxidant activity than those of the polyphenols they come from (Chen and Ho, 1997). The thermal decomposition products of rosmarinic acid, especially

DHPLA, would also contribute to the antioxidant activity. Moreover, the most common products of the lignocellulose thermal degradation process are reducing agents such as sugars and phenolic compounds (Hendriks and Zeeman, 2009). Therefore, the aggregated effects of formation and release of polyphenols derivates and non-polyphenolic compounds at high temperatures, would explain the negative correlation between antioxidant capacity assays and polyphenol subclasses analyzed by LC-MS. With the exception of DHPLA whose increase, similar to antioxidant activity, is due to degradation and hydrolytic reactions that occur at high temperatures.

2.4 Conclusions

Temperature and extraction time affected polyphenol extract profiles both quantitatively and qualitatively. High temperatures and high exposure times reduced the yield of phenolic compounds detected. One exception was the yield of hydroxyphenylpropanoic acid that reached a maximum at 200°C (the highest temperature in our study), probably due to the thermal degradation of rosmarinic acid. The clearest effect of increased exposure time occurred at 150 °C, at which flavonoids and hydroxycinnamic acids degradation and the extraction of hydroxyphenylpropanoic acids were favored. The extraction yield of flavones, flavonols/flavanones, hydroxycinnamic acids, and total polyphenols peaked at 100°C and 5 min.

In polyphenol PHWE from thyme, the quantity of neither total polyphenols nor any particular subclass of polyphenols identified in this work, with the exception of DHPLA, determined observed antioxidant activity, as the two factors returned strong negative correlations (inverse relationships) with the latter. Antioxidant activity increased steadily as temperature rose, whereas the quantity of polyphenols extracted decreased. Reactions occurring at temperatures above 100 °Caffected this behavior through polyphenol degradation and the formation and release of antioxidant-active nonpolyphenol compounds and polyphenol derivates such as DHPLA.

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3 EFFECT OF PRESSURIZED HOT WATER EXTRACTION ON ANTIOXIDANTS FROM GRAPE POMACE BEFORE AND AFTER FERMENTATION

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3.1 Introduction

Grapes (*Vitis spp*) are one of the largest fruit crops in the world (Sinha et al.) and are among the highest antioxidant-containing fruits (Speisky et al., 2012). In 2009, world grape production reached approximately 66.9 million tons, of which 71% corresponded to grapes for winemaking. Consequently, grape by-products are produced in massive quantities, especially by the winemaking industry. Pomace (skins, seeds, and stems) produced as a winery by-product, constitutes 20% of grapes by weight (Djilas et al., 2009). Pomace is a suboptimal crop fertilizer because of its inhibitory effect on plant seed germination due to the high polyphenol content, especially anthocyanins and condensed tannins (including pigmented polymers and non-pigmented proanthocyanidins) (Negro et al., 2003). However, these are valuable compounds that play significant roles in the health benefits (Diebolt et al., 2001) and sensory attributes of wine (González-Manzano et al., 2004).

Anthocyanins from grape skins are protective against diverse potentially damaging cellular oxidants through several biological mechanisms (Kong et al., 2003). Anthocyanins have also been used as natural food colorants for a long time (Jackman et al., 1987). Additionally, condensed tannins are one of the most abundant polyphenols in grapes (García-Marino et al., 2006), and many pharmacological and therapeutic effects of grape products such as antioxidant, anti-inflammatory, and antimicrobial activities, as well as cardioprotective, hepatoprotective, and neuroprotective effects have been primarily attributed to grape proanthocyanidin content (Nassiri-Asl and Hosseinzadeh,

2009). The antioxidant action of simple polyphenols has been questioned because of their low bioavailability (Tomás-Barberán and Andrés-Lacueva, 2012). Proanthocyanidins, which are not absorbed and remain in the gut due to their polymeric nature, may have direct effects on the stomach (Pastene et al., 2009) and intestinal mucosa, protecting these tissues from oxidative stress or carcinogen action (Manach et al., 2005). Therefore, even though most of the health benefits of wine have been attributed to polyphenols, it is not known how much of the grapes' original polyphenolic content remains in the pomace after fermentation in wine production. It is known, however, that fermentation favors the break down of the cell wall in grapes tissue (Ribéreau-Gayon et al., 2006). Hence, a clean and effective extraction process applied after fermentation should recover significant amounts of phenolic compounds. The analysis and chemical characterization of extractable grape pomace polyphenolic content before and after fermentation is a preliminary step in producing wines with enhanced health benefits and sensory attributes. Pomace hot water extraction techniques have minimal environmental impact, and are important processes for recovering grape polyphenolic extracts for use in the food, cosmetics, and pharmaceutical industries.

The first study objective was to investigate the effect of fermentation on the specific chemical content and extractability of grape pomace phenolic antioxidants. The second objective was to analyze the impact of extraction conditions on extract antioxidant activity and on the recovery of total antioxidants (TAs), principal polyphenols, anthocyanins, and tannins.

3.2 Materials and Methods

3.2.1 Chemicals

Reagents and standards used were analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, and sodium carbonate were purchased from Merck (Germany). Tripyridyl triazine (TPTZ), $FeCl_3(6H_2O)$, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,5-dihydroxybenzoic acid, ascorbic acid, gallic acid, maleic acid-sodium dodecyl sulfate, triethanolamine, iron(III) chloride, bovine serum albumin, sodium hydroxide, hydrochloric acid (37%), glacial acetic acid, and sodium chloride were obtained from Sigma (USA).

3.2.2 Grape pomace

Cabernet Sauvignon pomace was obtained from Carmen Vineyard, Region Metropolitana, Chile. The pre-fermentation process was performed at 18°C for 10 days and the must was loaded into a 10 m^3 fermentation tank. Fermentation was conducted between 25°C and 30°C for 21 days without pectolytic enzymes. Two samples of the same pomace were taken at different stages of the winemaking process. The first sample was taken at the beginning of the winemaking process just after the must was introduced into the fermentation tank (unfermented pomace). The second sample was taken after the fermentation process had finished (fermented pomace). Each sample was reduced to a particle size lower than 1-mm diameter by an Oster® blender (Sunbeam Products, Inc., Boca Raton, FL) and was frozen to -20°C until extraction.

3.2.3 PHWE

Both fermented and unfermented grape pomaces were subjected to PHWE. A 5 g sample (dry weight) of grape pomace was mixed with 100 g of neutral quartz sand to avoid filter clogging in the 100-mL stainless steel extraction cell. The grape pomace was extracted in an Accelerated Solvent Extraction device (ASE® 150, Dionex) with 50 mL of distilled and filtrated (0.22 µm) water to obtain a matrix/extractant ratio of 1:10. A full factorial design with two factors was performed in triplicate at 102 atm. The factors assessed were extraction temperature (50, 100, 150, and 200°C) and extraction time (5 and 30 min); these values were selected based on previous studies. After extraction, the cell contents were rinsed with 100 mL of distilled and filtrated (0.22 µm) water and purged for 360 s by applying pressurized nitrogen (10.2 atm). Finally, the collected extracts were freeze-dried and stored in amber vials at -20°C until analysis. Extract solutions of 1 g/L were prepared for analysis.

3.2.4 DPPH radical scavenging activity determination

Pomace extract anti-radical capacity was determined using the DPPH radical-scavenging method (Brand-Williams et al., 1995). Initially, a stock solution was prepared by dissolving 23.5 mg of DPPH reagent in 100 mL methanol. The DPPH working solution was prepared by further diluting (1:10 v/v) the stock solution with methanol. Fifty-microliter volumes of extract solutions at different concentrations were mixed with 2 mL DPPH working solution, which had been diluted on the day of use to a 50 µM final concentration, or as necessary to reach an absorbance of 0.8 units at 516 nm (DR 2000™ Spectrophotometer; Hach Company, Loveland, CO). Bleaching of DPPH was measured at 516 nm until the absorbance remained unchanged (~30 min; the kinetics of the extract/DPPH reaction were recorded to determine the time to reaction

stabilization) in the dark and at room temperature. The effective pomace extract concentration needed to inhibit 50% of DPPH radical absorption, half-maximal inhibitory concentration (IC_{50}; mg/L), was calculated. The extract antioxidant capacity was compared with Trolox, a synthetic hydrophilic vitamin E analogue, using the Trolox equivalent antioxidant capacity (TEAC) equation: TEAC = IC_{50} Trolox/IC_{50} sample (Clariana et al., 2011). DPPH values were expressed as mg of Trolox equivalent (TE) per gram of dry mass of pomace (dp).

3.2.5 FRAP determination

The FRAP test offers a putative index of antioxidant reducing capacity in a sample (Benzie and Strain, 1996). A working solution was prepared by mixing 300 mM of acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and a freshly prepared 20 mM $FeCl_3(6H_2O)$ solution in 10:1:1 (v/v/v) proportion. For the assay, 3 mL working reagent was mixed with 100 μL sample or calibration standard (ascorbic acid), and absorbance was measured at 593 nm after a 30 min reaction time (Pulido et al., 2000). A calibration curve was constructed using ascorbic acid (0.1-0.8 mM). The regression coefficient of ascorbic acid was 0.9989. Results were expressed as ascorbic acid equivalent (AAE) per gram of dp.

3.2.6 Total antioxidant (TA) determination by Folin assay

Given that Folin values systematically exceed the total polyphenol content values, the results obtained using the Folin-Ciocalteu assay (Singleton and Rossi, 1965) were considered in this work to represent TA values (Perez-Jimenez et al., 2010). A mixture of 4.25 mL phenolic extract (1 mg/mL) and 0.25 mL Folin-Ciocalteu reagent

were diluted 1:1 (v/v) with distilled water, and mixed with 0.5 mL a 10% sodium carbonate solution (w/v). Absorbance was measured at 765 nm after a 1 h reaction time at room temperature. A calibration curve was constructed using gallic acid as the calibration standard (20-90 mg/L). The regression coefficient of gallic acid was 0.9987. Results were expressed as gallic acid equivalent (GAE) per g of dp.

3.2.7 Polymeric pigments and tannin by Harbertson-Adams assay

Anthocyanins, condensed tannin, and polymeric pigment content in grape pomace extracts were determined with the Harbertson-Adams assay adapted from the A.E. Hagerman and L.G. Butler method (Hagerman and Butler, 1978). This method also yielded the contributions of small and large polymeric pigments (SPPs and LPPs, respectively). In the Harbertson-Adams assay, treating compounds with bisulfite, a bleaching compound, allows separation of monomeric pigments or free anthocyanins (compounds bleached by bisulfite) and total polymeric pigments (TPPs, compounds unable to be bleached by bisulfite). Because polymeric pigments are made up of tannins bound to anthocyanins, they should precipitate to some extent when treated with proteins. Therefore, treating these two groups of compounds with proteins separates them further into two new fractions: LPPs or those pigments that are precipitated with proteins and SPPs or those pigments that are not precipitated with proteins. Finally, the precipitation method used here is fast, highly reproducible, and can be used with either condensed or hydrolysable tannins. Purified extracts containing only polyphenolic components or crude extracts containing both phenolic and nonphenolic components can be analyzed with this technique (Hagerman and Butler, 1978). When the protein precipitation assay is used without bisulfite bleaching, the result is a mix of colored and non-colored species, called total tannins. Results were expressed as malvidin 3-*O*-glucoside equivalents per g of dp, catechin equivalents (CEs) per g of dp, and absorbance units for anthocyanins, total tannins, and polymeric pigments.

3.2.8 Qualitative proanthocyanidin analysis by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry (MS)

3.2.8.1 Sample conditioning.

The DHB matrix (2,5-dihydroxybenzoic acid, 10 mg) and the cationizing agent (sodium chloride, 1 mg) were dissolved in 1 mL 1% aqueous trifluoroacetic acid. One µL of this solution was mixed with 1 µL sample solution (1 mg lyophilized pomace extract dissolved in 1 mL of 1% aqueous trifluoroacetic acid), which was homogenized and deposited (2 µL) on a target plate. After drying at room temperature, the crystals were irradiated in the spectrometer (Mateos-Martin et al., 2012).

3.2.8.2 Analytical conditions.

A MALDI-TOF/TOF mass spectrometer (AutoFLEX™ III; Bruker Daltonics GmBH., Bremen, Germany) equipped with a pulsed N_2 laser (337 nm) controlled by the flexControl 1.1 software package (Bruker Daltonics) was used to obtain MS and tandem MS/MS data. The voltage was 20 kV and the reflectron voltage 21 kV. Spectra are the sum of 500 shots with a frequency of 200 Hz.(Mateos-Martin et al., 2012) The positive mode was chosen in agreement with the literature for these types of compounds (Monagas et al., 2010). Proanthocyanidin molecular weights were calculated according to the following equation: $[M + Na^+] = 290.08 \times EC + 274.08 \times AFZ + 306.07 \times EGC + 152.01 \times GAL - 2.02 \times B - 4.04 \times A + 22.99$ where EC, AFZ, EGC, and GAL correspond to the number of (epi)catechin, (epi)afzelechin, (epi)gallocatechin, and

galloyl moieties, respectively; and A and B correspond to the number of A and B linkages, respectively.

3.2.9 Statistical Analyses

Extractions and analyses were performed in triplicate with the data presented as mean value ± SD. Statgraphics® Plus for Windows, version 4.0 (StatPoint Technologies, Inc., Herndon, VA) was used for statistical analyses. To study the effects of fermentation stage, extraction temperature, and extraction time on overall extraction performance, analysis of variance (factorial) and least significant difference tests were applied to the response variables with p-values ≤ 0.05 considered indicative of statistically significant differences between comparator groups.

3.3 Results and Discussion

To have a common basis for comparison, results of all the analyses of the extracts are expressed in terms of dry mass of pomace (dp) before extraction.

3.3.1 Effect of fermentation and extraction temperature and time on total antioxidant recovery and antioxidant activity

Grape pomace antioxidant activity measured with the FRAP assay was only affected by extraction temperature ($p<0.001$). The FRAP value increased as the temperature increased, reaching a maximum at 150°C for unfermented pomace (4.4 mg

AAE/g dp) and 200°C for fermented pomace (4.6 mg AAE/g dp; Figure 3.1a). Similar studies have shown this positive effect of temperature on the reducing/antioxidant capability of plant extracts (Xu et al., 2008). Unexpectedly, for unfermented pomace a slight decrease in the reducing capacity with increasing temperature from 150°C to 200°C was observed. This decrease may be due to thermal degradation of reducing compounds, although this effect was not observed with fermented pomace. Fermentation processes degrade the pomace cell structure, increasing solubility of numerous pomace-derived compounds including polysaccharides, mannoproteins, seed cuticle, and certainly polyphenols, in both water and alcohol (Ribéreau-Gayon et al., 2006). Therefore, the type and amount of antioxidants extracted could be different when using fermented versus unfermented grape pomace.

Pomace extract antiradical activity assessed with the DPPH assay was significantly affected by the three factors assessed: fermentation, extraction temperature, and extraction time (all p-values <0.001). Fermented pomace extracted at 200°C for 5 min presented the highest value (184 mg TE/g dp). In most extraction conditions, fermented pomace showed higher antiradical activity than unfermented pomace (Figure 3.1b). Due to cell wall polysaccharides degradation, the extractability of phenolic compounds in the fermented pomace is enhanced (Pinelo et al., 2006), resulting in extracts with higher antiradical activity. Increasing extraction temperature enhanced antiradical activity, which peaked in the range between 150°C and 200°C (Figure 3.1b). The temperature effect was more pronounced at temperatures above 100°C, especially for fermented pomace. The positive effect of temperature on antioxidant PHWE from grape pomace has been reported previously (Aliakbarian et al., 2012). At 50°C and 100°C, time has no significant effect on extract antiradical activity. At 150°C, increased extraction time reduced extract antioxidant activity. Additionally, at 200°C, the antiradical activity of unfermented pomace extracts increased with time while that of fermented pomace extracts decreased. This shows that the antioxidant profiles of fermented and non-fermented pomace are different.

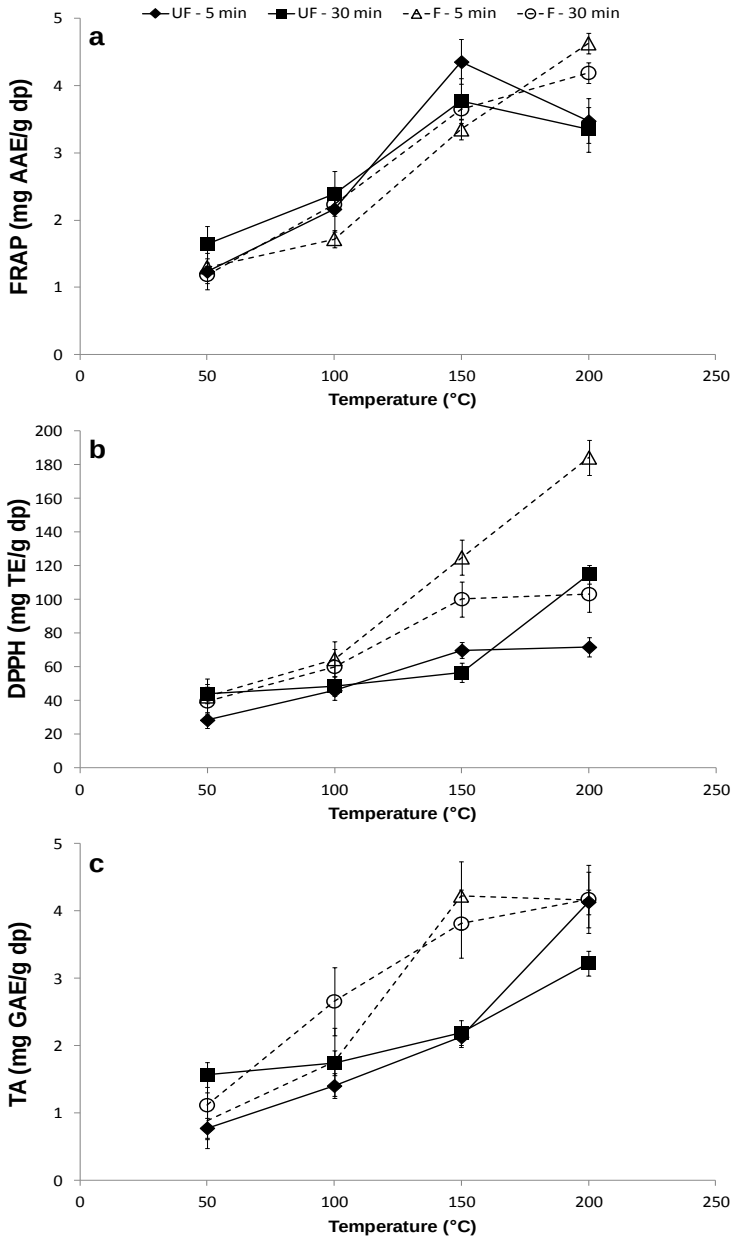

Figure 3.1 Effects of fermentation and extraction conditions on recovered total antioxidants and antioxidant activity in pressurized hot water extraction (PHWE) grape pomace extracts.

a) Ferric-reducing antioxidant power (FRAP) assay, b) 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and c) Folin assay. Curve symbols ♦ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols Δ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

Total antioxidant extraction was significantly affected by fermentation and temperature (both p-values <0.001). The maximum TA extraction yield was reached at 150°C and 5 min for fermented pomace (4.2 mg GAE/g dp) and at 200°C and 5 min for unfermented pomace (4.1 mg GAE/g dp). In most conditions, the fermented pomace extracts had the highest TA values, especially at high extraction temperatures (Figure 3.1c). Breakdown of grape skins and seeds during fermentation (Ribéreau-Gayon et al., 2006) facilitates the release and subsequent extraction of phenolic compounds, explaining the difference in quality and amount of antioxidants recovered from fermented versus unfermented pomace. Additionally, during high temperature extraction, cell wall degradation facilitates the release of other compounds, such as reducing sugars, which may alter the Folin assay measurement (Kumazawa et al., 2001), especially in materials with high sugar content such as unfermented pomace. The higher the extraction temperature the higher the TA value, which peaked between 150°C and 200°C (Figure 3.1c). The positive influence of temperature on the PHWE of polyphenols from grape pomace has been reported previously (Aliakbarian et al., 2012). Polarity, viscosity, and surface tension of water declines dramatically with increasing temperature, which enhances the capacity of water to solubilize and extract antioxidant compounds from pomace (Ong et al., 2006).

3.3.2 Effect of fermentation, and extraction temperature and time on anthocyanin extraction

The anthocyanin extraction yield is affected significantly by all three factors assessed, i.e., fermentation ($p<0.001$), extraction temperature ($p<0.001$), and extraction time ($p=0.001$). Figure 3.2a shows the effects of these factors, where the highest extraction yield was obtained for unfermented pomace extracted at 100°C for 5 min.

In most cases, higher anthocyanin yields were achieved from unfermented pomace. Anthocyanins are water-soluble pigments in the skin of red grapes and are

distributed in vacuoles that are covalently associated with pectins (Kennedy et al., 2001). Anthocyanins are extracted mainly in the aqueous phase during maceration prior to fermentation and at the beginning of alcoholic fermentation (Ribéreau-Gayon et al., 2006). Up to 77% of the anthocyanins are released in this process (Fournand et al., 2006). resulting in a residual pomace with low content of these pigments. Furthermore, within the anthocyanins family the structural differences between these compounds results in different extractabilities (Fournand et al., 2006). Therefore, anthocyanin profiles obtained from fermented and unfermented grape pomace should be different.

Regarding extraction conditions, an increase from 50°C to 100°C for 5 min increased anthocyanin extraction yield due to increased water solvation power and improved polyphenol solubility (Srinivas et al., 2009). However, at 150°C and 200°C, no anthocyanins were detected in the extracts. Moreover, an increase in extraction time decreased the amount of extracted anthocyanins, which was clearly observable at 100°C. Both temperature and exposure time have a strong influence on anthocyanin stability. Previous studies have reported that an arithmetic temperature increase causes a logarithmically-increased anthocyanin degradation (Rhim, 2002). Ju *et al* also reported that PHWE temperatures above 110°C decrease individual and total anthocyanins content in dried red grape skin extracts (Ju and Howard, 2003, 2005). Under excessive heat, grape pomace anthocyanins degrade by opening its pyrilium ring, thereby forming a colorless chalcone equivalent which further degrades to a brown insoluble polyphenolic compound, or by cleaving its sugar moiety to form a more labile anthocyanin aglycon (Mishra et al., 2008). In our study, these color changes were observed; from red (characteristic of the wine) in the extracts obtained at 50°C and 100°C to brown at 150°C and 200°C. Moreover, the influence of exposure time at high temperatures is very important in anthocyanin degradation. For example, during jam manufacture anthocyanins losses range between 10% and 80% when boiling time is varied from 10 to 15 min (García-Viguera and Zafrilla, 2001). Mishra *et al* reported that after 25 min at 126.7°C grape pomace anthocyanin degradation increased substantially (Mishra et al., 2008). Additionally, the formation of polymeric pigments (anthocyanins

bound to tannins) increases with temperature, especially at temperatures above 100°C, decreasing the amount of free anthocyanins (monomeric pigments) (Ju and Howard, 2003).

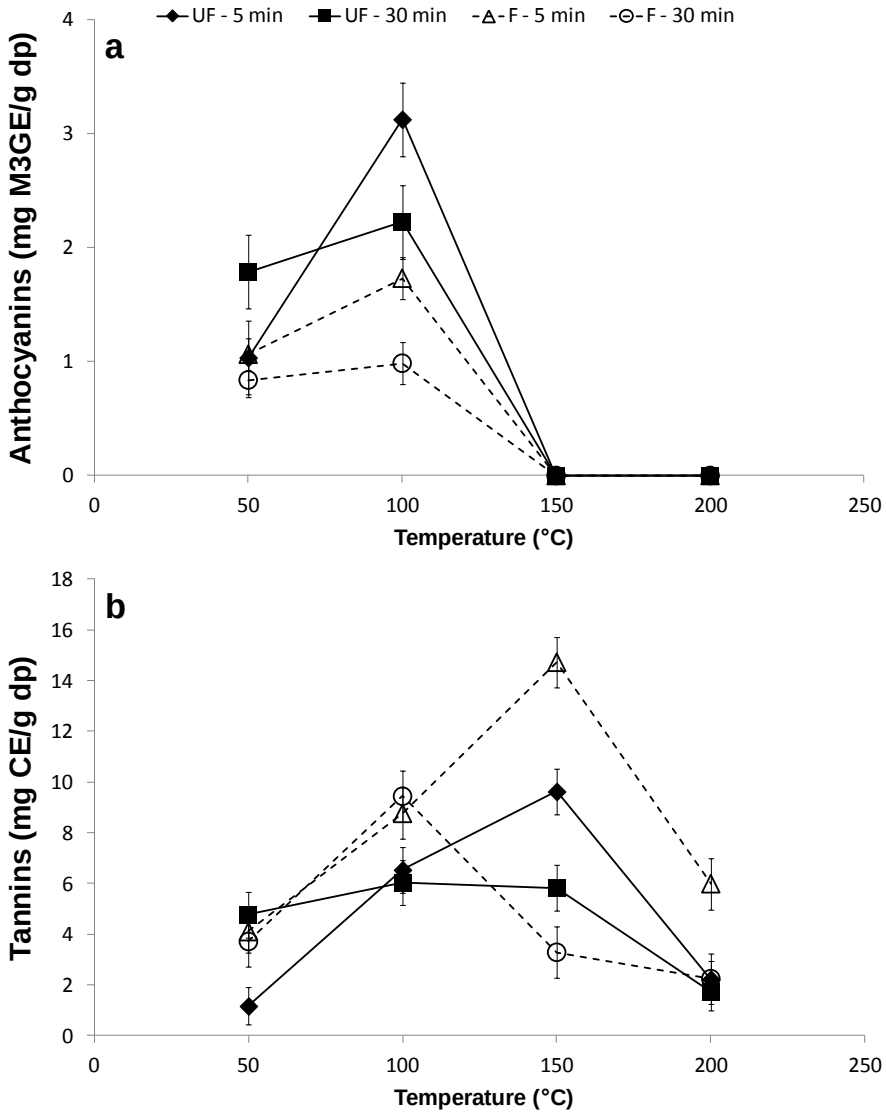

Figure 3.2 Effects of fermentation and extraction conditions on PHWE recovery of a) anthocyanins and b) condensed tannins from grape pomace.

Curve symbols ♦ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols Δ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

3.3.3 Effect of fermentation, and extraction temperature and time on tannin extraction

The tannin extraction yield is affected significantly by all three factors assessed: fermentation, and extraction temperature and time (all p-values <0.001). The effects of these factors are shown in Figure 3.2b, where the highest yield was obtained for fermented pomace extracted at 150°C for 5 min, followed by the unfermented pomace at the same extraction temperature and time.

In most of the extraction conditions tested (except 150°C and 30 min) the fermented pomace showed higher tannin yields than unfermented pomace. Only a small amount of tannins are released during fermentation, resulting in a fermented pomace with high tannin content and increased tannin extractability. Fournand *et al* reported that tannin extraction efficiency from unfermented grape skins in a hydroalcoholic solution similar to wine was lower than 38% (Fournand et al., 2006). Because tannin–cell wall interactions (hydrogen bonding and hydrophobic interactions) are determined by tannin and cell wall sugar structure and content (Hanlin et al., 2010). the cell wall degradation during fermentation and the PHWE operating conditions enhance tannin recovery. Additionally, tannins, depending on the degree of polymerization, have different extractabilities; a higher degree of polymerization equals a lower extraction yield during fermentation. Thus, in addition to the total amount of tannins, we expected that fermented and unfermented pomace extracts present different tannin structural profiles.

In most cases, higher tannin yields were achieved at 150°C and 5 min, while at 200°C the extraction yield greatly decreased from both unfermented and fermented pomace. In PHWE of tannins from grape seeds, where grape tannins are most concentrated,(Monagas et al., 2003) increasing the extraction temperature increases tannin extraction yield, peaking at 150°C (García-Marino et al., 2006). Likewise, Monrad *et al* found that the optimum temperature in the semi-continuous PHWE of grape pomace tannins is 140°C (Monrad et al., 2012). Extraction temperatures approaching 200°C may cause tannin degradation. To our knowledge, there are few

studies about tannin stability at temperatures above 100°C. However, it has been reported that the onset temperature of degradation of these polyphenols is approximately 150°C, and is dependent on factors such as acetylation and the amount of carbohydrates in the extract (Gaugler and Grigsby, 2009).

Varying the extraction time had different effects on tannin recovery, depending on the type of pomace (fermented or unfermented) and the extraction temperature. With unfermented pomace extracted at 50°C, increasing the extraction time from 5 to 30 min increased the tannin extraction yield. Because at low temperatures the tannin transfer rate is slower, increasing the extraction time results in higher yields. However, in the case of fermented pomace extracted at 50°C, the time increment produced no change in tannin extraction yield. Because the pomace was only partially degraded during fermentation, at low temperatures the mass transfer barriers within the matrix are strong, requiring a longer time to achieve solubility equilibrium. In contrast, at 150°C and 200°C increasing extraction time decreased tannin extraction efficiency for both unfermented and fermented pomace, because long exposure times to high temperatures favors polyphenol degradation (Gaugler and Grigsby, 2009).

We observed that tannins are more stable than anthocyanins at high temperatures. Similar results were found by Larrauri *et al* in a study of polyphenol stability during grape pomace skin drying, where tannins, perhaps due to their polymeric structure, were more stable than the total extractable polyphenols when exposed to temperatures between 100°C and 140°C (Larrauri et al., 1997).

3.3.4 Proanthocyanidin profiles observed by MALDI-TOF analysis

3.3.4.1 Compound identification

Tentative proanthocyanidin identification was performed by comparing the masses observed on mass spectra with the calculated mass for each compound. For identification, differences less than 0.3 Da between observed and calculated masses were considered acceptable. Proanthocyanidin results are summarized in Table 3.1.

The proanthocyanidins identified are mainly procyanidins and prodelphinidins with polymerization degrees up to 5 and 6, respectively. These findings are consistent with similar studies performed with positive ion reflectron mode where the polymerization degree ranged between 2 to 6 (Monagas et al., 2010). It should be considered that in grape seeds, highly polymerized procyanidins are generally more abundant than oligomers (Haslam, 1989). The detection limits of the MS and the poor extractability of these large polymers may hamper their identification.

Grape seeds possess significant amounts of procyanidins only, while grape skins and stems also contain (epi)gallocatechins units and therefore contain both B-type procyanidins and prodelphinidins (Vivas et al., 2004). Six procyanidins (B-type bond) were identified: a dimer and a trimer of (epi)catechin, and a dimer, trimer, tetramer, and pentamer of (epi)catechin with one gallate. These compounds have been found in grapes previously (Yang and Chien, 2000). Twelve compounds were identified from the prodelphinidin family: two dimers (A-type bond) with one and two (epi)gallocatechin(s); one dimer (B-type bond) with two (epi)gallocatechins; oligomers from dimer to hexamer (B-type bond) with one (epi)gallocatechin; and oligomers (B-type bond) from dimer to hexamer with one (epi)gallocatechin, all with one gallate. Prodelphinidins trimers up to hexamers has been previously reported in grapes (Vivas et al., 2004).

3.3.4.2 Effect of extraction temperature and time on proanthocyanidins profile

Fermentation, extraction temperature and extraction time caused striking changes in the grape pomace extract proanthocyanidin profiles (

Table **3.1**). Procyanidin dimers and trimers were found only in fermented pomace extracts. In fermented extracts, prodelphinidins and procyanidins with one gallate present higher polymerization degrees than those found in unfermented extracts. Additionally, more procyanidins and prodelphinidins with one gallate were identified in the extracts from fermented versus unfermented grape pomace. Propelargonidins were detected mainly in fermented pomace at high extraction temperatures (100 and 150°C). During fermentation, lower proanthocyanidin extraction yields, especially those with a high degree of polymerization, are observed (Fournand et al., 2006). These differences in extractabilities result in fermented pomace extracts with different proanthocyanidins profile than unfermented extracts. Moreover, degradation of the cell wall during fermentation(Ribéreau-Gayon et al., 2006) facilitates extraction of a greater variety of proanthocyanidins.

Most of the proanthocyanidins detected by MALDI-TOF were recovered at 50°C and 100°C. Additionally, the highest proanthocyanidin yields and polymerization degrees, from both fermented and unfermented pomace extracts, were found after 100°C and 5 min extractions. Higher extraction temperatures and times dramatically reduced the number of proanthocyanidins detected. At 150°C only a few proanthocyanidins were detected, and at 200°C no proanthocyanidins were recovered. Flavanol monomers and polyphenol degradation products were not detected in these extracts, possibly due to hydrolysis or limitations of the MALDI-TOF analysis. It has been reported that temperatures above 100°C favor the formation of polymeric pigments (tannins bond to

anthocyanins) (Ju and Howard, 2003), which could also explain why proanthocyanidins were not detected in our high-temperature extracts.

Table 3.1 Proanthocyanidins in grape pomace extracts obtained with different pressurized hot water extraction (PHWE) conditions, as analyzed and identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry

Proanthocyanidin Subclass	**Compound**	**FW (obsd)**	**Na adduct (calcd)**	**°C**	**50**		**100**		**150**	
				min	**5**	**30**	**5**	**30**	**5**	**30**
Procyanidins	Dimer (B)	601.1	601.0		**F**		**F**		**F**	
	Trimer (B)	889.3	889.1		**F**	**F**	**F**	**F**		
	Dimer (B – 1 GE)	753.2	753.0		**U/F**	**U/F**	**U/F**	**U/F**	**F**	**F**
	Trimer (B – 1 GE)	1041.3	1041.1		**U/F**	**U/F**	**U/F**	**U/F**		
	Tetramer (B – 1 GE)	1329.4	1329.2		**U/F**	**U/F**	**U/F**	**U**		
	Pentamer (B – 1 GE)	1617.4	1617.2			**F**	**F**			
Prodelfinidins	Dimer (A – 1 EGC)	615.1	615.0				**U**			
	Dimer (A – 2 EGC)	631.1	631.0				**U**			
	Dimer (B – 1 EGC)	617.1	617.0		**U/F**	**U**	**U**			
	Trimer (B – 1 EGC)	905.3	905.1		**U/F**	**U/F**	**U/F**	**U/F**		
	Tetramer (B – 1 EGC)	1193.3	1193.1		**U/F**	**U/F**	**U/F**	**U/F**		
	Pentamer (B – 1 EGC)	1481.4	1481.2		**U/F**	**U/F**	**U/F**	**U**		
	Hexamer (B – 1 EGC)	1769.3	1769.3			**F**	**F**			
	Dimer (B – 1 EGC – 1 GE)	769.1	769.0		**F**	**F**	**F**			
	Trimer (B – 1 EGC – 1 GE)	1057.2	1057.1		**U/F**	**U/F**	**U/F**			
	Tetramer (B – 1 EGC – 1 GE)	1345.2	1345.2		**U/F**	**U/F**	**U/F**			
	Pentamer (B – 1 EGC – 1 GE)	1633.2	1633.2		**U**	**U/F**	**U/F**			
	Dimer (B – 2 EGC)	633.1	633.0		**U/F**	**U/F**	**U**	**U**	**U**	

3.3.5 Effect of fermentation, temperature and time on polymeric pigments

Polymeric pigments consist of anthocyanins (monomeric pigments) bound to tannins or flavan-3-ols, such as catechin or epicatechin that are formed in wine after fermentation (Harbertson et al., 2003). The stability of these pigments depends on various factors: the type of molecule, the concentration of the solution, pH, temperature, oxidation, light exposure, and the types of solvents. Therefore, any experimental data detailing how extraction reactions affect and modify the main polyphenol content in grape pomace are valuable.

Fermentation, and extraction temperature and time significantly affected recovery of SPP, LPP, and TPP (all p-values <0.001). The highest SPP extraction yield was obtained in unfermented pomace extracted at 150°C for 30 min, whereas the highest LPP and TPP extraction yields were obtained in fermented pomace extracted at 150°C for 5 min.

In most cases, unfermented pomace showed higher SPP values than fermented pomace, especially with 30 min extraction times (Figure 3.3a). In contrast, higher LPP and TPP yields were obtained with fermented pomace (Figure 3.3b and Figure 3.3c). LPP and TPP showed similar extraction yield patterns because the LPP group is the largest contributor to TPP. In the Harbertson-Adams assay, LPPs represent the colored fraction of the condensed tannins; hence these two values are directly related (Harbertson et al., 2003). Fermented pomace gives higher tannin extraction yields that are representative of higher LPP values.

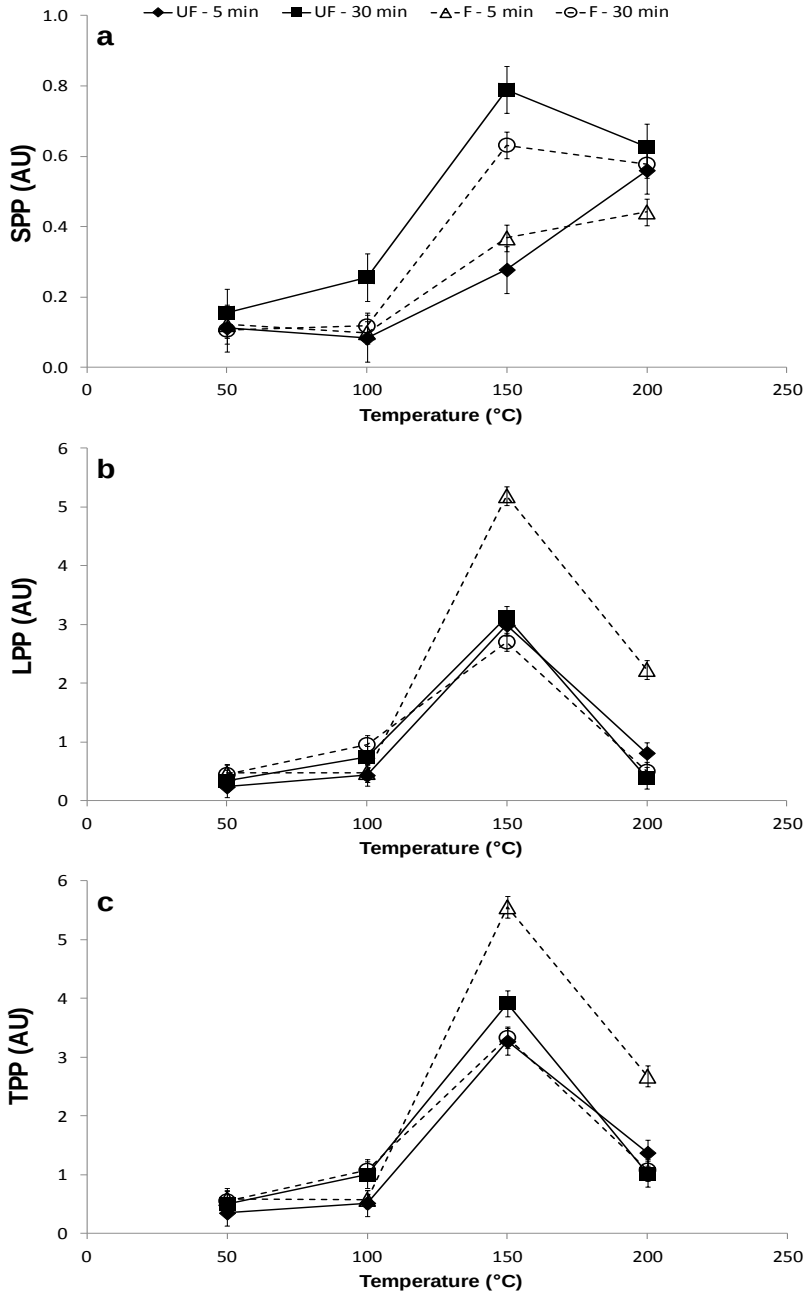

Figure 3.3 Effects of fermentation and extraction conditions on PWHE recovery of a) small polymeric pigment (SPP), b) large polymeric pigment (LPP), and c) total polymeric pigment (TPP) from grape pomace.

Curve symbols ♦ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols Δ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

Increasing extraction temperature increased the recovered SPP and LPP, and therefore the TPP values, peaking at 150°C, and markedly decreasing at 200°C (except for SPP extracted for only 5 min). Increased extract polymeric color with increasing extraction temperature, especially at temperatures above 100°C, has been reported previously (Ju and Howard, 2003). The contribution of polymers to extract color indicates that extensive "degradation" of anthocyanins at high extraction temperatures occurred either by thermal degradation or polymeric pigment formation (Ju and Howard, 2003). Extraction time showed no clear impact on polymeric pigment recovery from grape pomace. The highest SPP values were found at extractions times of 30 min in most conditions tested. Longer extraction times; hence, high anthocyanin-tannin reaction times, could favor the formation of SPPs at low temperatures and the breakdown of LPPs at high temperatures, increasing the SPP content in both cases. In contrast, longer extraction times have negative (especially at high temperatures) or no effect on LPP and TPP yields, possibly due to thermal degradation (Gaugler and Grigsby, 2009).

3.3.6 Correlation between polyphenol subclasses, total antioxidants, and antioxidant activity

Several different assays were statistically correlated using the Pearson correlation coefficient (Table 3.2). We calculated correlations after separating the data according to treatment (unfermented or fermented) and extraction time (5 or 30 min). Analysis of antioxidant activity and TA showed a strong positive correlation that was more pronounced with fermented pomace. This observation agrees with previous findings in polyphenol PHWE (Budrat and Shotipruk, 2009). However, both antioxidant activity and TA showed strong negative correlations with total recovered anthocyanins, especially for fermented pomace. This is expected because anthocyanins are degraded at temperatures above 100°C, while antioxidant activity as well as TA increased with temperature and peaked at 150°C to 200°C. At extraction times of 5 min, total tannins

showed weak positive correlations with antioxidant activity and TA, while at 30 min these correlations were negative. Increased extraction time decreases tannin extraction efficiency at high temperatures due to thermal degradation (Gaugler and Grigsby, 2009), while in most cases the extraction time has little or no effect on antioxidant activity and TAs.

Table 3.2 Pearson product-moment correlation coefficient between the different assays of antioxidants recovered during grape pomace PHWE.
Correlation coefficients were calculated with the values of all extraction temperatures separated by treatment (unfermented or fermented) and extraction time (5 or 30 min)

Assays	U		F	
	5 min	30 min	5 min	30 min
FRAP-DPPH	0.953	0.529	0.999	0.991
FRAP-Folin	0.651	0.689	0.931	0.987
DPPH-Folin	0.840	0.979	0.918	0.977
FRAP-Anthocyanins	-0.596	-0.870	-0.849	-0.894
DPPH-Anthocyanins	-0.563	-0.675	-0.821	-0.926
Folin- Anthocyanins	-0.557	-0.795	-0.859	-0.825
FRAP-Tannins	0.637	-0.202	0.240	-0.430
DPPH-Tannins	0.451	-0.919	0.213	-0.465
Folin-Tannins	-0.088	-0.839	0.576	-0.284

U, unfermented pomace; F, fermented pomace; FRAP, ferric-reducing antioxidant Power; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay

The amount of the main polyphenols extracted from unfermented and fermented grape pomace possesses negative or weak correlations with respective antioxidant activity and TA, mainly due to the high temperature effects. In most cases, an increase from 50°C to 100°C produced an increase in extract polyphenol content, antioxidant activity, and TA, whereas a temperature increase to 100°C or 150°C decreased recovered polyphenol content but increased antioxidant activity and TA. It is possible that the formation of other compounds with antioxidant capacity (i.e., degradation, depolymerization, and polymerization products) has an important contribution to the heat-induced altered relationship between the main polyphenolic compounds and antioxidant activity and TA.

Polymerization and depolymerization phenomena could change the "availability" of OH groups, thereby increasing or decreasing the antioxidant capacity of the resulting compounds. At 150°C hydrolysis of galloylated compounds increases gallic acid content of grape extracts (García-Marino et al., 2006), which may also increase antioxidant activity. Furthermore, excessive heating results in thermal degradation of the catechins and procyanidins to derived products such as catechinic acid (Fuleki and Ricardo-da-Silva, 2002), additionally modifying extract antioxidant capacity.

Increasing PHWE temperature from 100°C to 160°C linearly increases antioxidant activity, but extraction temperatures above 110°C decrease individual and total anthocyanins contents (Ju and Howard, 2005). This decrease could be due to thermal degradation of target molecules as well as formation of polymeric pigments. Chalcone glycosides, phenolic acids, and phloroglucinaldehyde are the major anthocyanin thermal degradation products. However, the loss of anthocyanin antioxidant activity was compensated by the antioxidant capacity of newly formed phenolics after heating (Sadilova et al., 2007). Conversely, in aqueous systems at high temperatures many plant material reactions produce antioxidants or reducing compounds. The high antioxidant activity of extracts obtained at 150°C and above may be associated, in part, with the generation of antioxidant-capable Maillard reaction products such as melanoidins (Plaza et al., 2010a). Grapes also contains smaller amounts of other

polyphenols such as stilbenes and flavonols (Nassiri-Asl and Hosseinzadeh, 2009), which, despite being degraded at high temperatures, may contribute to antioxidant activity either by themselves or through their degradation products (Vergara-Salinas et al., 2012).

3.4 Conclusions

The PHWE of antioxidants from fermented grape pomace, in most of the extraction conditions tested, allows recovery of a greater amount of TAs and antioxidant activity equivalent than from unfermented pomace. In both fermented and unfermented pomace, the highest antioxidant recoveries were obtained at temperatures above 150 °C. Although the majority of anthocyanins were removed during fermentation, high amounts of anthocyanins were recovered from fermented grape pomace using moderate temperatures (100°C) and short extraction times. Contrary to anthocyanins, high extraction temperatures (about 150°C) and short times yielded higher amounts of tannins. Extraction temperature determined the proanthocyanidin profile, different in fermented and unfermented pomace, where the greatest amount of these compounds was recovered at lower temperatures (50 and 100°C). Overall, we found that grape pomace antioxidant activity and TA were not directly related to the main polyphenol content in PHWE extracts. The data obtained here in a laboratory-scale equipment will be useful to develop an industrial scale PHWE processes.

List of abbreviations used

TA, total antioxidant; PHWE, pressurized hot water extraction; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPTZ, tripyridyl triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; IC50; half-maximal inhibitory concentration; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox equivalent; FRAP, ferric-

reducing antioxidant power; AAE, ascorbic acid equivalent; GAE, gallic acid equivalent; TPP, total polymeric pigment; SPP, small polymeric pigment; LPP, large polymeric pigment; CE, catechin equivalent; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; DHB, 2,5-dihydroxybenzoic acid

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4 CHARACTERIZATION OF PRESSURIZED HOT WATER EXTRACTS OF GRAPE POMACE: CHEMICAL AND BIOLOGICAL ANTIOXIDANT ACTIVITY

The contents of this chapter was submitted to the journal

Food Chemistry

4.1 Introduction

Diverse phenomena highly dependent on temperature occur during the PHWE of polyphenols from plant materials: polyphenols degradation and formation of polyphenols-derived antioxidants, selective polyphenol extraction, depolymerization, and polymerization (Ju and Howard, 2005; Vergara-Salinas et al., 2013), as well as the generation of antioxidant-potent Maillard reaction products (MRPs) such as melanoidins (Plaza et al., 2010a) and hydroxymethylfurfural (He et al., 2012). In PHWE the relationship between polyphenols content and antioxidant activity of the extracts is low or even inverse (Vergara-Salinas et al., 2013). Therefore, depending on the PHWE temperature used, it is possible to obtain extracts with different compositions and antioxidant activities and, consequently, different activities in biological systems.

The ability of chemical antioxidant activity assays to predict the *in vivo* activity is questioned because it does not consider several physiological aspects, such as bioavailability and metabolism alterations (Liu and Finley, 2005). However, cell cultures are suitable to assess the potential action of the antioxidants in a biological system, previous to animal and human studies (Liu and Finley, 2005). The human promyelocytic leukemia line, HL-60, has been widely used to study oxidative stress-related aspects. This cell line is very sensitive to oxidative stress inducers such as ultraviolet radiation and oxygen peroxide (Verhaegen et al., 1995) and it is a

good option for preliminary evaluation of the potential protective effect of antioxidant compounds.

It has been reported that grape polyphenols in cell line culture ranges reduce oxidative stress, inhibit DNA damage induced by reactive oxygen species (Apostolou et al., 2013) and activate the antioxidant response (Xia et al., 2010). However, diverse and even contrary performance of polyphenols described in the literature (Xia et al., 2010) evidence the complex and still unclear action of the phenolic antioxidants in biological systems. Polyphenolic antioxidants can inhibit the formation of reactive oxygen species in mammalian cell cultures (Wei et al., 1995) but also exhibit a dose-dependent prooxidant toxicity (Sergediene et al., 1999). For example, the treatment of tubule cells (LLC-PK_1) with grape polyphenols, especially oligomers, had potent protective effects against high glucose-induced oxidative stress (Fujii et al., 2006). However, it has been reported that several toxic substances can be formed by the oxidation of phenolic compounds added to cell culture media (Halliwell, 2008). Anthocyanidins, through the production of reactive oxygen species, induce the apoptosis in HL-60 cells (Hou et al., 2004). This complexity increases in the case of crude extracts (mixtures of many compounds) and it could be even higher in the pressurized hot water extracts due to the degradation and formation of new compounds (e.g. MRPs) at high temperatures. MRPs can suppress oxidative stress and inflammation in human cells cultures (Kitts et al., 2012). However, these compounds are considered to be toxic and mutagens (Husøy et al., 2008).

The objective of this study was to assess grape pomace extracts obtained at different extraction temperatures in terms of polyphenols and MRPs content, chemical antioxidant activity, and bioactivity on HL-60 cell line culture under oxidative conditions, including protective effect on cell growth and mitochondrial membrane potential.

4.2 Materials and Methods

4.2.1 Chemicals and cell line

Reagents and standards used were analytical grade. Folin-Ciocalteu reagent, methanol and sodium carbonate were purchased from Merck (Germany). Tripyridyl triazine (TPTZ), $FeCl_3(6H_2O)$, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,5-dihydroxybenzoic acid, ascorbic acid, gallic acid, maleic acid-sodium dodecyl sulfate, triethanolamine, iron(III) chloride, bovine serum albumin, sodium hydroxide, hydrochloric acid (37%), glacial acetic acid, and sodium chloride, 5-hydroxymethylfurfural (5-HMF), RPMI-1640, D-glucose, L-glutamine and Human promyelocytic leukemia cells HL-60 were obtained from Sigma (USA). Heat inactivated fetal bovine serum was obtained from Gibco (Brazil). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1) was obtained from Life Technologies (USA). Valinomycin was obtained from Santa Cruz Biotechnology (USA).

4.2.2 Grape pomace

Cabernet Sauvignon pomace was obtained from Carmen Vineyard, Region Metropolitana, Chile. The pre-fermentation process was performed at 18°C for 10 days and the must was loaded into a 10 m^3 fermentation tank. Fermentation was conducted between 25°C and 30°C for 21 days without pectolytic enzymes. The grape pomace sample was taken after the fermentation process had finished. Each sample was reduced to a particle size lower than 1-mm diameter by an Oster® blender (Sunbeam Products, Inc., Boca Raton, FL) and then frozen to -20°C until extraction.

4.2.3 Pressurized hot water extraction

Grape pomace was subjected to pressurized hot water extraction (PHWE). A 5 g sample (dry weight) of grape pomace was mixed with 100 g of neutral quartz sand to avoid filter clogging in the 100-mL stainless steel extraction cell. The grape pomace was extracted in an Accelerated Solvent Extraction device (ASE® 150, Dionex) with approximately 50 mL of distilled and filtered (0.22 µm) water to obtain a matrix/extractant ratio of 1:10. The extractions were made in triplicate during 5 minutes at two temperatures: 100 (GPE100) and 200°C (GPE200). After extraction, the cell contents were rinsed with 100 mL of distilled and filtered (0.22 µm) water and purged for 360 s by applying pressurized nitrogen (10.2 atm). Finally, the collected extracts were freeze-dried and stored in amber vials at -20°C until analysis. Extract solutions of 1 g/L were prepared for analysis.

4.2.4 Ferric-reducing antioxidant power determination

The Ferric Reducing Ability of Plasma (FRAP) test offers a putative index of antioxidant reducing capacity in a sample (Benzie and Strain, 1996). A working solution was prepared by mixing 300 mM of acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and a freshly prepared 20 mM $FeCl_3(6H_2O)$ solution in 10:1:1 (v/v/v) proportion. For the assay, 3 mL working reagent was mixed with 100 µL sample or calibration standard (ascorbic acid), and absorbance was measured at 593 nm after a 30 min reaction time (Pulido et al., 2000). A calibration curve was constructed using ascorbic acid (0.1-0.8 mM). The regression coefficient of ascorbic acid was 0.9989. Results were expressed as ascorbic acid equivalent (AAE) per gram of dry extracts (d.e.).

4.2.5 Total antioxidant determination by Folin assay

Total antioxidants were determined by Folin assay. Although this method is commonly considered for polyphenol analysis, it indeed determines all compounds in the sample with antioxidant capacity and not only polyphenols (Perez-Jimenez et al., 2010). A mixture of 4.25 mL phenolic extract (1 mg/mL) and 0.25 mL Folin-Ciocalteu reagent was diluted 1:1 (v/v) with distilled water, and mixed with 0.5 mL a 10% sodium carbonate solution (w/v). Absorbance was measured at 765 nm after 1 h reaction time at room temperature. A calibration curve was constructed using gallic acid as the calibration standard (20-90 mg/L). The regression coefficient of gallic acid was 0.9987. Results were expressed as gallic acid equivalent (GAE) per g of d.e.

4.2.6 Anthocyanins and tannin determination by Harbertson-Adams assay

Anthocyanins and condensed tannin content in grape pomace extracts were determined with the Harbertson-Adams assay adapted from the Hagerman and Butler method (Hagerman and Butler, 1978). Results were expressed as malvidin 3-*O*-glucoside equivalents per g of dp, catechin equivalents (CEs) per g of d.e., and absorbance units for anthocyanins, total tannins, and polymeric pigments, respectively.

4.2.7 Determination of Maillard reaction products (MRPs) by Absorbance measurement

Maillard reaction products (melanoidins) were estimated by means of browning intensity of the grape pomace extracts. These extracts were filtered and the browning intensity was directly measured at 360 and 420 nm (Plaza et al., 2010a).

4.2.8 Determination of Phenolic Compounds and Hydroxymethylfurfural content

Phenolic compounds ((+)-catechin, (-)-epicatechin, kaempferol and myricetin) and hydroxymethylfurfural (5-HMF) were measured on a HPLC system consisting of a 1,024 photodiode-array detector model L-2455, a high throughput analysis pump model L-2130, a column oven model L-2350 and an autosampler model L-2200 (Hitachi LaChrom Elite, Japan). The extracts were re-suspended in a methanol/water (50/50) solution at 20 mg/mL. After injecting 30 µL of sample, the separation was performed using a reverse phase Nova-Pack C18 column (300 mm x 3.9 mm i.d. 4 µm) at 20 °C (Waters Corp.). Two mobile phases were employed for elution: A (water/acetic acid, 98:2, v/v) and B (water/acetonitrile/acetic acid, 78:20:2, v/v/v). The gradient profile was 0-55 min, 100-20% A and 0-80% B; 55-57 min, 20-10% A and 80-90% B; 57-90 min, 10% A and 90% B isocratic, followed by washing with methanol and re-equilibration of the column (Peña-Neira et al., 2000). The flow rate was 1.0 mL/min from 0 to 55 min and 1.2 mL/min from 55 to 90 min. Detection was performed by scanning from 220 to 600 nm with an acquisition speed of 1 s. Identification and quantification were carried out by comparison (spectrum and retention time) with the standard molecule.

4.2.9 Cell culture, growth conditions and treatments

Human promyelocytic leukemia cells HL-60, were obtained from Sigma-Aldrich (Sigma, 98070106). The cells were grown in a RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum, 2 g/L D-glucose and 2 mM L-glutamine. Cells were grown in CO_2 incubator (Forma Scientific, USA) at 37°C in an atmosphere of 5 % CO_2 with 95 % humidity. HL-60 cells were suspended at 2.5 x 10^5 cell/mL and cultured in 12-well plates (Orange Scientific). To assess antioxidative capability, HL-60 cells

were incubated with 0.15 and 0.015 mg/mL of grape extract, using 0.1 mM H_2O_2 as positive control of oxidative environment. As control of antioxidative capability, HL-60 cells were treated with 0.15 mM Trolox, using 0.1 mM H_2O_2.

4.2.10 Loss of mitochondrial membrane potential

A cell sample from a growth assay taken at 24 h was used to detect the loss of mitochondrial membrane potential ($\Delta\psi_m$). HL-60 cells were incubated with 10 µg/mL JC-1 at 37°C. Loss of $\Delta\psi_m$, was assayed by observing a shift in fluorescence emission from red (~590 nm) to green (~525 nm) using flow cytometry (Beckman Coulter Cytimics, FC 500) (Prince et al., 2008). HL-60 cells were treated with 100 nM valinomycin as a positive control of membrane potential loss (approximately 98%).

4.2.11 Statistical analysis

Extractions and analyses were performed in triplicate with the data presented as mean value ± SD. Statgraphics® Plus for Windows, version 4.0 (StatPoint Technologies, Inc., Herndon, VA), was used for statistical analyses. Tests were applied to the response variables with p-values ≤ 0.05, which were considered to be indicative of statistically significant differences between comparison groups; these tests allow us to study the effects of extraction temperature, extract composition and activity, factorial variance analysis and least significant difference.

4.3 Results and Discussion

4.3.1 Characterization of the extracts

The extracts were characterized in terms of antioxidant activity (AA), total antioxidant content (TAC), polyphenol content, and content of Maillard reaction products (MRPs) and 5-HMF in particular (Table 4.1), in an attempt to relate their composition to their protective antioxidant bioactivity in HL-60 cell culture.

4.3.1.1 Antioxidant activity and total antioxidants content

The grape pomace extracts obtained at 100°C (GPE100) and at 200°C (GPE200) showed significant differences in their AA and TAC. GPE200 exhibited 50% more AA and 30% more TAC than GPE100 (Table 4.1). These results are in agreement with the findings of similar research in which the TAC of the extracts and their AA increased with the extraction temperature (Plaza et al., 2010a).

In the PWHE of plant materials, temperature has a positive effect on the extract's TAC and on its ability to reduce antioxidants; this is due to several chemical reactions that occur at high temperatures. The thermal degradation of cell wall facilitates the release of phenolic cell wall-associated compounds (Pérez-Jiménez and Torres, 2011) resulting in extracts with higher antiradical activity. It may also release reducing sugars, which may alter the Folin assay measurement (Kumazawa et al., 2001). Furthermore, several studies have reported the formation of compounds with high antioxidant capacity (e.g. melanoidins) from the Maillard reaction in extraction processes at high temperatures (Aliakbarian et al., 2012; Plaza et al., 2010a)

Table 4.1 Characterization of the pressurized hot water extracts from grape pomace[a]

Extract		GPE100	GPE200
FRAP (mg AAE/g d.e.)		10.2[b]	15.0[c]
Total Antioxidants (mg GAE/g d.e.)		10.6[b]	13.6[c]
Anthocyanins (mg M3GE/g d.e.)		10.5	n.d.
Condensed tannins (mg EC/g d.e.)		52.9[b]	18.3[c]
(+)-Catechin (mg/g d.e.)		0.81[b]	0.65[c]
(-)-Epicatechin (mg/g d.e.)		1.05[b]	1.24[c]
Kaempferol (mg/g d.e.)		0.12[b]	0.03[c]
Myricetin (mg/g d.e.)		0.18[b]	0.16[c]
Resveratrol (mg/g d.e.)		0.02	n.d.
MRPs by Absorbance (AU)	360 nm	0.318[b]	0.371[c]
	420 nm	0.148[b]	0.281[c]
HMF (mg 5-HMF/g d.e.)		n.d.	0.087

[a]Values with the same letter (b-c) in each row showed no statistically significant difference at the confidence interval of 95%. M3GE, malvidin 3-*O*-glucoside equivalents; d.e., dry extract; EC, epicatechin equivalents; FRAP, ferric-reducing antioxidant power; AAE, ascorbic acid equivalents; GAE, gallic acid equivalents; MRPs, Maillard reaction products; AU, absorbance units; HMF, Hydroxymethylfurfural; n.d., no detected

4.3.1.2 Absorbance measurement of MRPs

The GPE200 presented significantly higher content of MRPs than GPE100 based on the browning measurement at 360 and 420 nm (Table 4.1). These absorbance values are employed as an indicator of caramelization and formation of brown advanced MPRs in thermally processed foods. Our data show an increase in the formation of MRPs with the increase of the extraction temperature in the PHWE of grape pomace. The formation of MRPs during the PHWE of several plant samples has also been reported by other researchers (He et al., 2012; Plaza et al., 2010a). The MRPs includes a wide range of compounds of significant importance for the nutritional value of food and beverages. Some of these compounds present strong antioxidant activities but others such as hydroxymethylfurfural (5-HMF) could be toxic and mutagenic (Husøy et al., 2008).

4.3.1.3 5-HMF content

5-HMF was detected only in GPE200 with a concentration of 0.087 mg per g of extract (Table 4.1). The formation of 5-HMF is favored at high temperatures and at pH 7 or below (Martins et al., 2000). The increase in the extraction temperature produced a decrease in the pH of the extracts due to a decrease in the amino groups available as Maillard reaction progress (Liu et al., 2008). Plaza et al. (2010a) reported pH values between 5 and 7 in pressurized hot water extracts from several plant samples.

4.3.1.4 Polyphenols content

The extracts evaluated showed a large difference in their polyphenols content (especially anthocyanin and tannin content) due to thermal degradation. The total polyphenol content of GPE100 was about three times higher than GPE200. In the GPE200, no anthocyanin was detected and its tannins content was considerably lower than GPE100. Its content of (+)-catechin, kaempferol and myricetin were also lower than GPE100, whereas its (-)-epicatechin content was slightly higher (Table 4.1).

Temperature has a strong influence on polyphenols stability, especially on anthocyanins. In water at temperatures of 100°C and above simple flavonoids are degraded and the formation of derived antioxidant compounds is favored. Previous studies have reported that PHWE temperatures above 110°C decrease individual and total anthocyanins content in grape skin extracts (Ju and Howard, 2005). Antioxidant compounds such as chalcone glycosides, phenolic acids, and phloroglucinaldehyde are the major anthocyanin thermal degradation products (Sadilova et al., 2007). To our knowledge, there are few studies of tannin stability at temperatures above 100°C. However, Gaugler and Grigsby (2009) reported that the onset temperature of degradation of these polyphenols is approximately 150°C, and it is dependent on factors such as the acetylation and content of carbohydrates in the extract . The breakdown of tannins at 200°C (with the consequent release of subunits) could be the reason for the higher (-)-epicatechin content in GPE200. Tannins, according to our results, are more stable than simple flavonoids at high extraction temperatures. The high thermal stability of tannins compared to the other polyphenols may be due to their polymeric structure (Larrauri et al., 1997).

4.3.2 Bioactivity of the grape pomace extracts

The protective antioxidant bioactivity of the GPE100 and GPE200 extracts were assessed in HL-60 cell culture under oxidative conditions given by the presence of an oxidant agent (H_2O_2). Their activity was compared with the protective activity of Trolox (the reference antioxidant compound) at 24 h of cell growth. Both extracts exhibited potent protective activities on HL-60 cell growth and mitochondrial membrane potential ($\Delta\psi_m$), comparable to the activity of Trolox, but also showed cytotoxic activity.

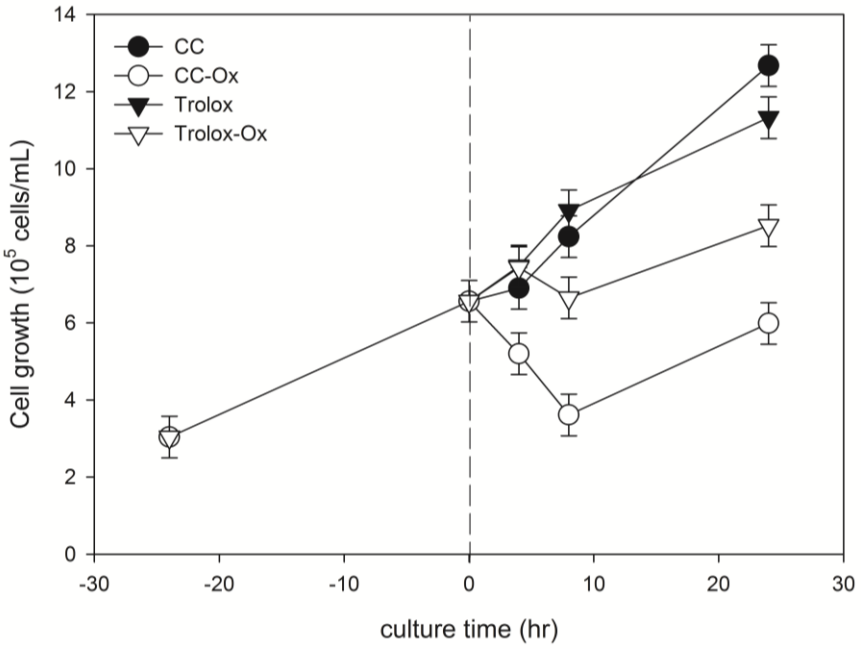

Figure 4.1 Evaluation of protective effect of Trolox on HL-60 cells growth (concentration of viable cells) under oxidative conditions.

Control culture (CC) in optimal growth conditions and culture under oxidative conditions, 0.1 mM H_2O_2 (Ox), are used as reference curves to evaluate the protective effect of Trolox. Trolox: culture with 0.15 mM Trolox, and Trolox-Ox: culture with 0.15 mM Trolox and 0.1 mM H_2O_2. Bars represent the upper and lower limits of the 95% confidence interval.

In the presence of the oxidant, Trolox and both extracts at the highest concentration assessed (0.15 mg/mL) exhibited protective antioxidant activity on cell growth of HL-60. Trolox showed the highest protective effect increasing the cell growth in 42%, compared to the control culture with the oxidant (Figure 4.1). Likewise, GPE100 and GPE200 increased the cell growth in 27% and 24% respectively (Figure 4.2 and 4.3). Although both crude extracts possess different composition, they showed a similar protective effect. The level of this protective activity was close to that exhibited by Trolox despite the low purity of the grape polyphenolic extracts. At the lowest concentration (0.015 mg/mL) no effect on HL-60 growth was observed.

The positive effect of the extracts on the $\Delta\psi_m$ was observed both in the absence and in the presence of the oxidant (Figure 4.4). In the absence of the oxidant, GPE100 and GPE200 showed a protective effect at both concentrations, decreasing the $\Delta\psi$m loss to values lower than that of the control culture, especially at the highest concentration. Trolox showed no effect on $\Delta\psi$m in the absence of the oxidant. In the presence of the oxidant, the HL-60 cells showed a $\Delta\psi_m$ loss three times higher than those of the control culture. This negative effect of the oxidant was strongly inhibited by both GPE100 and GPE200 at 0.15 mg/mL, reaching values of $\Delta\psi_m$ loss of 1% and 5% respectively- which are lower than those of Trolox and the control culture. At 0.015 mg/mL no effect on $\Delta\psi_m$ of HL-60 was observed.

GPE100 present higher content of polyphenols, especially anthocyanins and condensed tannins, than GPE200, which may explain its greater protective effect against oxidation. The lipid peroxidation induced by oxidative stress causes the loss of $\Delta\psi_m$ and the release of cytochrome c into the cytosol, triggering the caspase cascade leading to the apoptotic cell death in HL-60 cells (Anuradha et al., 2001). Some phenolic compounds prevent the decrease in mitochondrial membrane potential and suppress the accumulation of reactive oxygen species (Guo et al., 2005). Condensed tannins or proanthocyanidins from grape have shown a protective effect on mitochondrial membrane reducing and restoring the $\Delta\psi_m$ altered by oxidative stress (Li et al., 2004).

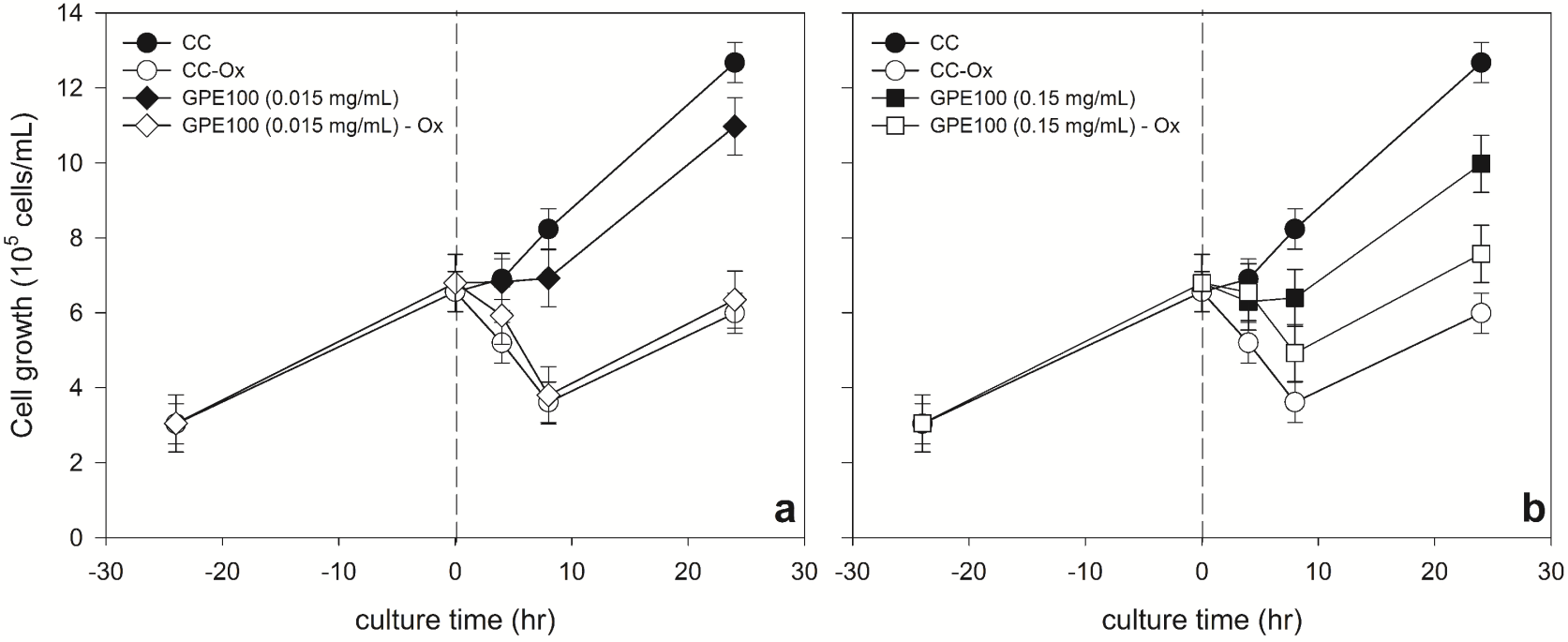

Figure 4.2 Evaluation of protective effect of GPE100 at two concentrations on HL-60 cells growth under oxidative conditions.

Control culture (CC) in optimal growth conditions and culture under oxidative conditions, 0.1 mM H_2O_2 (Ox), are used as reference curves to evaluate the protective effect of the extract. **a**) HL-60 cell culture treated with GPE100 at 0.015 mg/mL and GPE100 at 0.015 mg/mL plus 0.1 mM H_2O_2 (Ox). **b**) HL-60 cell culture treated with GPE100 at 0.15 mg/mL and GPE100 at 0.15 mg/mL plus 0.1 mM H_2O_2 (Ox). Bars represent the upper and lower limits of the 95% confidence interval.

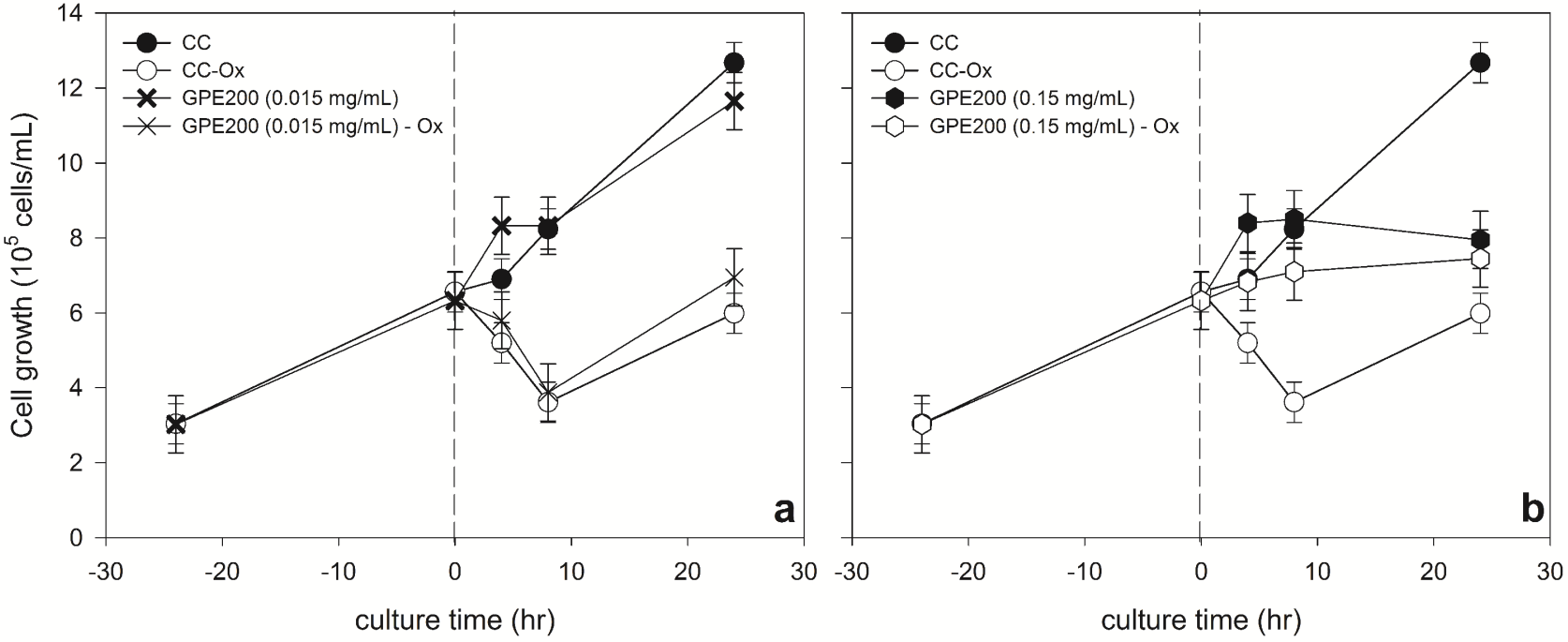

Figure 4.3 Evaluation of protective effect of GPE200 at two concentrations on HL-60 cells growth under oxidative conditions.

Control culture (CC) in optimal growth conditions and culture under oxidative conditions, 0.1 mM H_2O_2 (Ox), are used as reference curves to evaluate the protective effect of the extract. **a**) HL-60 cell culture treated with GPE200 at 0.015 mg/mL and GPE200 at 0.015 mg/mL plus 0.1 mM H_2O_2 (Ox). **b**) HL-60 cell culture treated with GPE200 at 0.15 mg/mL and GPE200 at 0.15 mg/mL plus 0.1 mM H_2O_2 (Ox). Bars represent the upper and lower limits of the 95% confidence interval.

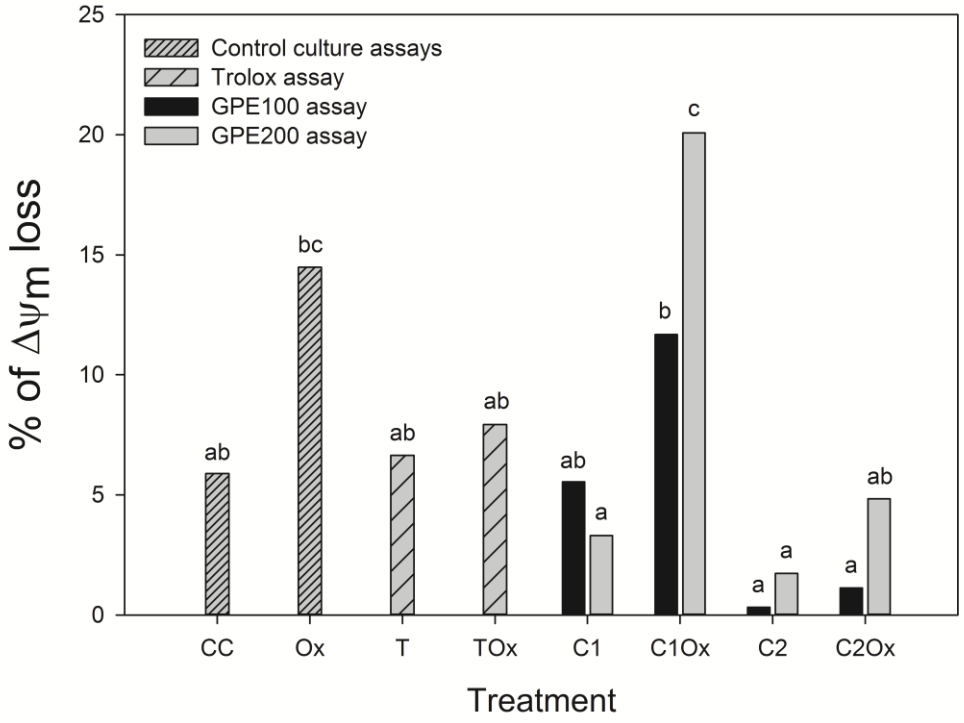

Figure 4.4 Evaluation of protective effect of GPE100 and GPE200 at two concentrations on mitochondrial membrane potential (Δψm) of HL-60 cells under oxidative conditions compared with the protective effect of Trolox.

Control culture (CC) in optimal growth conditions and culture under oxidative conditions, 0.1 mM H_2O_2 (Ox), are used as reference curves to evaluate the protective effect of Trolox and extracts. T: culture with 0.15 mM Trolox, TOx: culture with 0.15 mM Trolox and 0.1 mM H_2O_2, C1: culture with GPE100 or GPE200 at 0.015 mg/mL, C1Ox: culture with GPE100 or GPE200 at 0.015 mg/mL and 0.1 mM H_2O_2, C2: culture with GPE100 or GPE200 at 0.15 mg/mL, C2Ox: culture with GPE100 or GPE200 at 0.15 mg/mL and 0.1 mM H_2O_2. Bars with the same letter showed no statistically significant difference at the confidence interval of 95%.

Additionally, the grape extracts (also Trolox) showed no negative effect on the $\Delta\psi_m$ in the absence of the oxidant (Figure 4.4) despite the oxidized forms of polyphenols may cause mitochondrial toxicity by collapsing the $\Delta\psi_m$. However, this negative effect is highly dependent on the concentration. For example, Wang et al. (1999) reported that

increasing the concentration of apigenin, quercetin, myricetin, and kaempferol increases the loss of $\Delta\psi_m$, the reactive oxygen species production, the release of mitochondrial cytochrome c into the cytosol and the caspase activity. Therefore, the concentrations assessed in this work produced no negative effect on the $\Delta\psi_m$.

Conversely, Trolox, GPE200 and GPE100 showed cytotoxic effect on the HL-60 cell growth in the absence of the oxidant. Trolox decreased the cell growth in about 11% compared to the control culture (Figure 4.1). At the lowest concentration (0.015 mg/mL), only a slight cytotoxic effect of GPE100 was observed, reducing the growth in about 13% (Figure 4.2). At 0.15 mg/mL, GPE100 and GPE200 showed a significant negative effect on the cell growth; however, the strength of their cytotoxic effect was different, GPE100 reduced the growth in 21% while GPE200 reduced the growth in 37% (Figure 4.2 and 4.3). The higher cytotoxic effect exhibited by GPE200 may be due to its higher MPRs content and the presence of 5-HMF. Additionally, plant polyphenols have shown anticancer activity inhibiting the growth of different cancer cell lines (Stagos et al., 2012). For example, polyphenolic grape stem extracts inhibit at low concentrations the growth of HepG2 and HeLa, with activities comparable to those of grape seed extracts (Apostolou et al., 2013). The results presented in this article along with previous work in the area would suggest the use of these bioactive extracts and their principal bioactive compounds as cancer preventive agents.

Finally, these results illustrate the complex action of antioxidants in a biological system. Polyphenols and other antioxidants could present protective antioxidant or prooxidant/cytotoxic activities in cell cultures depending on several factors such as their concentration, their ability to oxidize, their lipophilicity, the content of other antioxidants and metals, and the oxidative stress level of the cell culture (Halliwell, 2008; Kitts et al., 2012; Sergediene et al., 1999).

4.4 Conclusions

Our results evidence the effects of extraction temperatures on the chemical composition, antioxidant activity and bioactivity of pressurized hot water extracts. High extraction temperatures (200°C) yield extracts (GPE200) with higher AA, higher MPRs and lower polyphenols content than extracts obtained at 100°C (GPE100). In addition, 5-HMF was only detected in GPE200 extracts. Under oxidative conditions, both extracts exhibited a high protective bioactivity on HL-60, similar or higher than that exhibited by Trolox. Under normal culture conditions, GPE200 exhibited higher cytotoxicity on HL-60 cancer cells than GPE100, possibly due to their higher MRPs content.

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5 FINAL CONCLUSIONS AND FUTURE DIRECTIONS

PHWE is a promising green extraction process for obtaining polyphenols (and other phytochemicals) from plant matrices. Using pressurized hot water as solvent presents several advantages over traditional extraction methods. Unlike commonly used solvents, water is non-flammable, non-toxic, readily available and environmentally friendly. Its solvating properties can be profoundly improved by increasing the temperature, which enhance the aqueous solubility of polyphenols and the diffusion process. This enhancement results in high extraction yields at short extraction times compared to conventional solvent extractions. According the results found in the PHWE of deodorized thyme and grape pomace, simple flavonoids (flavones, flavonols, flavanones and anthocyanins) and phenolic acids in general reach a maximum extraction yield at 100°C. Higher temperatures (above 100 °C) and longer exposure times produce the degradation of these compounds. Polymeric flavonoids (condensed tannins) are maximally extracted at 150°C evidencing high thermal stability, may be due to their polymeric structure.

In the PHWE of polyphenols from plants matrices, higher temperatures (above 100 °C), besides polyphenol degradation, also would favour the formation and release of polyphenol derivatives, non-polyphenolic antioxidants from Maillard reaction, and potentially toxic and mutagenic compounds with high antioxidant activity such as hydroxymethylfurfural. Therefore, increasing extraction temperature results in a low extraction yield of polyphenols but high recovery of total antioxidants.

The extract obtained at moderate extraction temperature (100 °C) present a high polyphenols content, whereas the extracts obtained at higher temperatures (200 °C) possess higher antioxidant content but lower polyphenols.

The grape pomace extract obtained at 100 °C show higher polyphenols content but lower total antioxidants content than the extracts obtained at 200 °C. Conversely, both extracts protect similarly the growth of mammalian cells (HL-60) under oxidative

conditions, showing an activity comparable to that exhibited by Trolox (antioxidant of reference). The extract obtained at 200 °C however showed a high cytotoxicity in the absence of oxidant, probably due to its high content of Maillard compounds. These results support the potential use of the highly antioxidant extracts as a protection against the oxidative damage. However, the greater antioxidant activity of the extracts also results in higher cytotoxicity evidencing the complex and disputed action of antioxidant on biological systems. Some antioxidants (polyphenolic and non-polyphenolic) also could be prooxidant and toxic under specific biological condition. Then, further research on bioactivity of the principal antioxidants in the extracts is necessary to identify their positive or negative effects and their adequate form of application. Consequently, the extracts obtained at high temperature processes such as PHWE possibly cannot be used without post-treatment. Further purification processes should be applied for obtaining safe products.

Finally, the knowledge presented in this thesis along with further research is essential to control the PHWE process, improve the quality of the extract and define the requirements for further purification processes.

Further research in our laboratory is focused on: i) the experimental analysis and modeling of the water solubility and thermal degradation of fundamental polyphenols, such as flavanols and phenolic acids, ii) the identification and isolation of the antioxidants (polyphenolic and non-polyphenolic) present in pressurized hot water extracts to evaluate their antioxidant and biological activity and iii) concentration and purification of polyphenolic extracts. Also, we consider a requirement to deepen on the main causes and dynamics of formation of neo-antioxidants compounds.

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