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PhD Thesis

Antioxidant properties of extracts from selected plant materials (*Caesalpinia spinosa*, *Perilla frutescens*, *Artemisia annua* and *Viola wittrockiana*) *in vitro* and in model food systems

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“Let food be thy medicine,

and let medicine be thy food”

Hippocrates

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ABSTRACT

Phenolic compounds, ubiquitous in plants, are of considerable interest and are increasingly becoming a subject of intensive research due to their bioactive properties such as antioxidant, antimicrobial, anti-mutagenic, anti-viral and anti-inflammatory activity. The objective of this research was to determine the antioxidant activity of extracts from selected plant materials, namely *Caesalpinia spinosa*, *Perilla frutescens*, *Artemisia annua* and *Viola wittrockiana* Gams. Plant material extracts were studied by *in vitro* methods, such as Total Phenolic Content using Folin Ciocalteu reagent, the measurement of scavenging capacity against the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation, the oxygen radical absorbance capacity (ORAC), and the ferric reducing antioxidant power (FRAP).

Many *in vitro* methods, such as ABTS, ORAC or FRAP assay, have been developed to evaluate antioxidant activity. Unfortunately, these methods often correlate poorly with the ability to inhibit oxidative deterioration of foods because the *in vitro* assays do not account for factors such as the physical location of the antioxidant, its interaction with other food components, and environmental conditions. To evaluate accurately the potential of antioxidants in foods, models must be developed that resemble conditions expected in food products. This study outlines model systems for the evaluation of natural antioxidants in two types of food: oil-in-water emulsions and meat model systems.

In addition, in all analyzed samples the content of the main phenolic compounds were determine using techniques such as high performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS).

The extract of *Caesalpinia spinosa* was tested for its antimicrobial effect against some common microorganisms and for growth promoting properties with respect to probiotic *Lactobacillus plantarum* strain.

The results of this research indicate that extracts from the plants studied may be suitable for use as natural food additives.

Keywords: Antioxidants; free radical scavenging; emulsion; meat; lipid oxidation; phenolics

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AAPH	2,2’azobis(2-amidinopropane) dihydrochloride
ABTS	2,2’ azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)
AUC	Area under the curve
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CE	Catechin equivalents
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FRAP	Ferric Ion Reducing Antioxidant Powder
FW	Fresh weight
GAE	Gallic acid equivalents
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
ME	Malvidin glucoside equivalents
ORAC	Oxygen Radical Absorbance Capacity
PCA	Principal Component Analysis
RE	Rutin equivalents
ROS	Reactive Oxygen Species
TBA	Thiobarbituric acid
TBARs	2-thiobarbituric acid reactive substances
TEAC	Trolox Equivalent Antioxidant Capacity
TAC	Total Antocyanin Content
TFC	Total Flavonoid Content
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
TPTZ	2,4,6-tripyridyl-s-triazine

1 INTRODUCTION

1.1 General Introduction

Phenolic compounds, ubiquitous in plants, are of considerable interest and have received more and more attention in recent years due to their bioactive functions. Polyphenols are amongst the most desirable phytochemicals because of their antioxidant activity. These components are known as secondary plant metabolites and possess also antimicrobial, antiviral and anti-inflammatory properties along with high antioxidant capacity (Ignat, Volf, & Popa, 2011; Santas, Almajano, & Carbó, 2010).

Lipid oxidation is a serious problem in foods because it produces rancid odours and flavours, decreases shelf life, alters texture and colour, and decreases nutritional value (Waraho et al., 2012). For example, lipid oxidation has been found to be one of the major causes of quality deterioration in processed muscle foods (Brewer, 2011). Food emulsions are another example of a food that can rapidly degrade by lipid oxidation reactions (Poyato et al., 2013). Numerous methods have been developed to control the rate and extent of lipid oxidation in foods, one of the most effective being the addition of antioxidants. In brief, an antioxidant is a synthetic or natural compound that has the ability to slow down lipid oxidation when present at low concentration compared to an oxidisable lipid. Most commercial food antioxidants work by scavenging free radicals or chelating metals. Free radical scavengers, such as tocopherols, butylated hydroxytoluene (BHT), and plant phenolics, inhibit lipid oxidation by reducing peroxyl and alkoxyl radicals to stable compounds. Through these pathways, free radical scavengers can inhibit chain propagation and formation of fatty acid decomposition products (e.g., aldehydes and ketones) that cause rancidity (Alamed, Chaiyasit, McClements, & Decker, 2009). In the food industry, the attention of manufacturers has shifted from synthetic to natural antioxidants as, although so far the synthetic antioxidants have been economically used to control effectively oxidation and prolong the shelf life of foods, their effectiveness and safety have been questioned due to their high volatility and instability at elevated temperatures and their suspected carcinogenicity when consumed at excessively high levels of intake (Ramful et al., 2011).

In evaluating the potential antioxidant functions of components in natural plant extracts as prophylactic agents or food additives, it is important to employ a number of analytical

techniques since the antioxidant potency can differ substantially according to the physical and chemical parameters of the systems used for their characterization (Zhou & Elias, 2013). It is also important to assess fully the levels of active phenolic components present in crude extracts and the interaction of plant extracts with other antioxidants in order to understand comprehensively the antioxidant mechanism of natural polyphenols in food.

1.2 The Background to the Research Problem

In relation to food, antioxidants were originally defined as "substrates that in small quantities are able to prevent or greatly retard the oxidation of easily oxidisable nutrients such as fats" (Skibsted, 2010). Antioxidants can prevent oxidative damage to food during processing, storage and preparation of meals. Antioxidants may accordingly help the development of more healthy food with low levels of lipid and protein oxidation products. Antioxidants may also have more direct health effects as part of the diet, but methodological problems in assessing this have been identified since both vitamin antioxidants (vitamin E and C) and non-vitamin antioxidants (polyphenols and carotenoids) are multifunctional in biological systems and cannot be evaluated by "one-dimensional" methods (Frankel & Meyer, 2000).

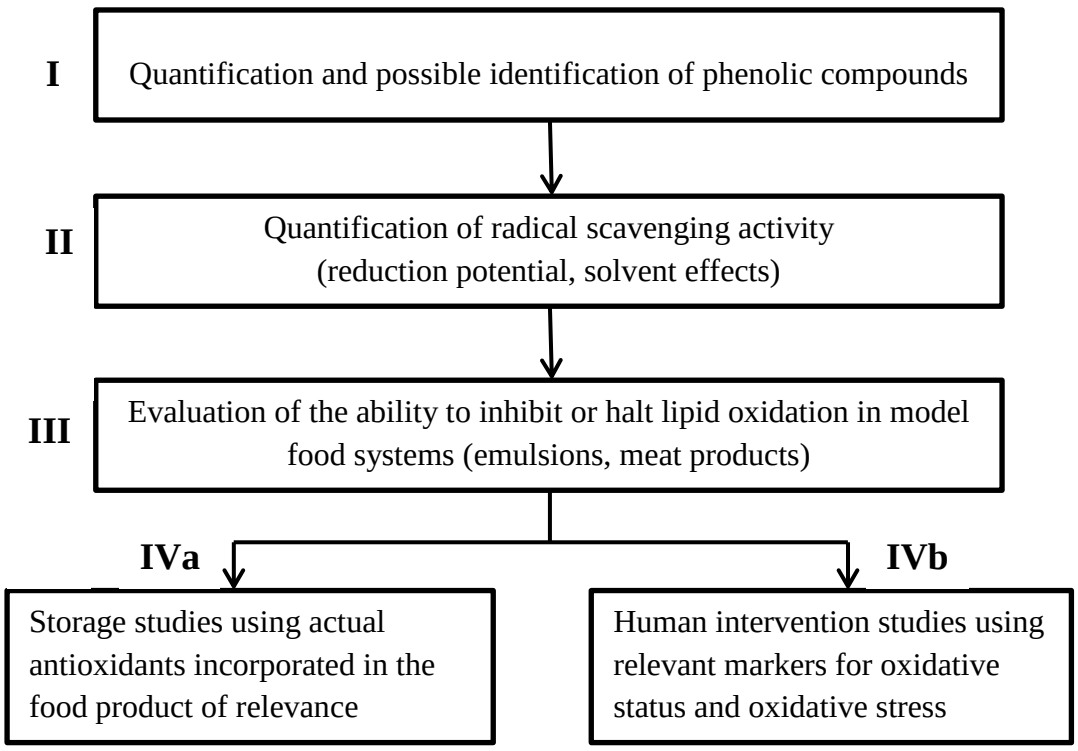

Figure 1.1 Antioxidant evaluation strategy as proposed by Becker et al. (2004).

A four-step strategy for antioxidant evaluation has been proposed (Becker, Nissen, & Skibsted, 2004). As seen from Fig. 1.1, the final evaluation of antioxidants depends on storage experiments using antioxidants for food protection, and on human intervention studies for health effects of antioxidants. Most standard assays used for antioxidant evaluation deal with antioxidants as reducing agents or as scavengers of radicals (Wolfe & Liu, 2007). Screening of potential antioxidants for radical scavenging capacity or reducing activity using simple assays corresponding to step I (quantification), step II (radical scavenging) or step III (effects in model systems) to predict protective effects on food stability or health effects in humans does not seem scientifically justified. Quantification of radical scavenging capacity or reducing activity alone provides only guidelines for the final evaluation in storage experiments or in human intervention studies (Lund, Hviid, & Skibsted, 2007).

1.3 The Statement of the Research Problem

Various synthetic or natural antioxidants can be used in order to prevent oxidative reactions in food products. However, because of consumer concern about the potential health hazards associated with dietary intake of synthetic antioxidants, the focus of this study was to employ plant phenolic compounds as natural antioxidants.

1.4 The Hypothesis

The hypothesis is that extracts from selected plant materials, namely *Caesalpinia spinosa* (tara), *Perilla frutescens, Artemisia annua* and *Viola wittrockiana* Gams. are a source of highly effective antioxidants and anti-microbial molecules, and are suitable for use as natural food additives.

2 Objective of the Research

The objectives of the study were to:

- Find the best extraction method to take advantage of the antioxidant properties of components in selected plant materials namely *Caesalpinia spinosa*, *Perilla frutescens, Artemisia annua* and *Viola wittrockiana* Gams.
- Determine the antiradical capacity of plant extracts and evaluate the effects of these extracts in oil-in-water emulsions.
- Evaluate the effect of tara pods on the shelf life of model meat systems.
- Evaluate the antimicrobial activity of tara pod extracts.
- Evaluate the effect of tara pod extracts on the growth of probiotic *Lactobacillus plantarum* 8014 strain.
- Determine the content of the main phenolic compounds in plant extracts using techniques such as high performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS).

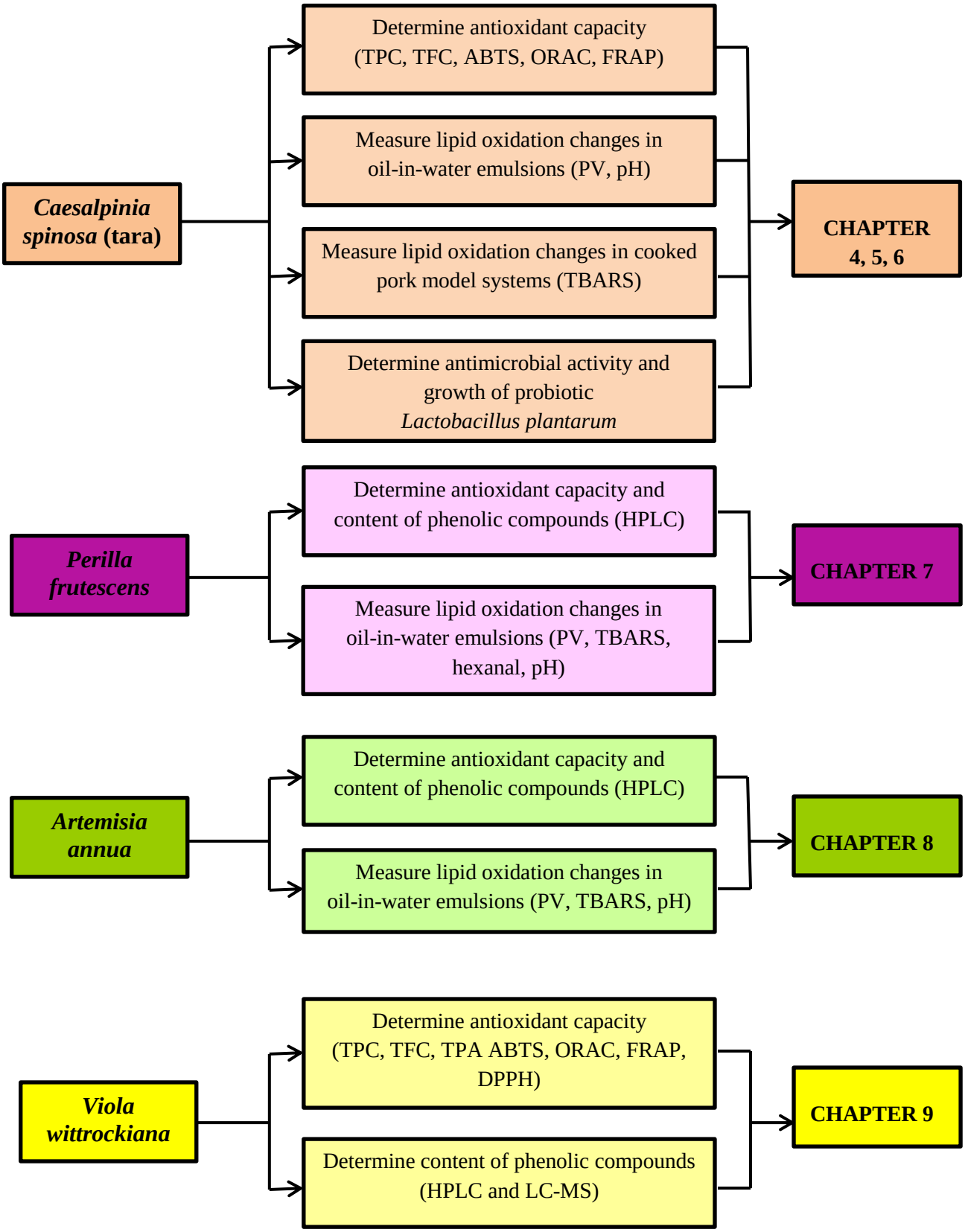

Figure 2.1 Study of the antioxidant effects of plant material (*in-vitro* and in model food systems).

3 LITERATURE REVIEW

3.1 Antioxidants in plants

Plants are well known to produce a diverse array of secondary metabolites to engage with the world around them. The major classes of secondary metabolites are alkaloids, terpenes, and phenols. Phenols are typically classified as either low molecular weight simple phenols, or as high molecular weight polyphenols. The quantity and type of polyphenols created by plants vary considerably between species (El Gharras, 2009).

Polyphenols became ubiquitous in the plant kingdom for protection of plants against UV radiation. Polyphenols further provide a repair mechanism for plants through oxidative polymerization by enzymes following mechanical damage. These compounds play an important role in plant growth and reproduction, providing an efficient protection against pathogens and predators, besides contributing to the colour, sensory characteristics and nutritional properties of fruits and vegetables (Naczk & Shahidi, 2006).

Given the abundance of polyphenols in the typical human diet, and the potential bioactivities of phenols, their biological impact has been actively explored in the past decade. Some polyphenols are proposed as therapeutic agents for a variety of diseases or to promote general health (Škerget et al., 2005).

3.1.1 Roles of antioxidants in food and human health

In the past few decades, there has been growing evidence that oxidative stress and specific human diseases can be prevented by including in the diet plant foods that contain large amounts of antioxidants such as vitamins C, E or natural antioxidants such as flavonoids, tannins, coumarins, phenolics and terpenoids (Perumalla & Hettiarachchy, 2011).

Dietary antioxidants can act as free radical scavengers, radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors (Karadag, Ozcelik, & Saner, 2009). Therefore, there is increasing interest in extending the range of antioxidants that can be used as food ingredients to prevent food oxidation. Furthermore, phenolic extracts prepared from plant materials (such as green tea, grape seed, aromatic herbs) are known to have antimicrobial effects against foodborne pathogens (Almajano, Carbo, Jimenez, & Gordon, 2008; Perumalla & Hettiarachchy, 2011).

Antioxidants such as BHT, BHA and plant extracts have been widely used as additives, preservatives or supplements in foods (Zulueta, Esteve, Frasquet, & Frígola, 2007). Ou et al. (2002) suggested that increasing the intake of dietary antioxidants may help to maintain the antioxidant status and normal physiological functions of the human body. Although antioxidants are recognized as important phytonutrients, presently, there is no recommended daily "total antioxidant" intake recommended due to the diversity and complexity of antioxidants (Kaliora, Dedoussis, & Schmidt, 2006). Thus, *in vitro and in vivo* studies on the antioxidant properties and effects of foods such as fruit and vegetables are still required.

Health diseases such as heart disease, macular degeneration, diabetes and cancer are all influenced by cellular oxidative damage. There has been increasing interest in the mechanism of action of antioxidants and whether they specifically intercept or remove free radicals from cells in the human body. Ames et al. (1993) reported that antioxidants prevent injury to blood vessel membranes, optimize blood flow to the heart and brain, prevent cancer-causing DNA damage, and lower the risks from cardiovascular and Alzheimer's diseases. Jo et al. (2006) also indicated that antioxidants can prevent or slow the oxidative damage linked to various diseases such as carcinogenesis, atherogenesis and aging. Additionally, the flavan-3-ols from cocoa have been reported to have a vasodilatory effect which improved blood flow (Faridi et al., 2008). Chlorogenic acid, which is present in high amounts in coffee, is reported to have beneficial effect on cardiovascular disease (Bonita, Mandarano, Shuta, & Vinson, 2007) and to decrease the risk of type II diabetes (Bidel, Hu, & Tuomilehto, 2008).

It is suggested that all these diseases might be retarded or prevented by protective compounds which have the ability to inhibit reactive oxygen species (ROS) formation, scavenge free radicals, or chelate metals (Panteleon et al., 2008). In the body, natural endogenous antioxidant systems have been developed to deal with the production of free radicals and have been divided into enzymatic and non-enzymatic groups. Examples of the enzymatic antioxidants are superoxide dismutase, gluthathione peroxidase and catalase (Rojas & Brewer, 2008) and non-enzymatic antioxidants are β-carotene, vitamin C, and vitamin E. There are also phytochemical antioxidants, such a polyphenols, lycopene and lutein that can also protect the body from oxidation damage (Moon & Shibamoto, 2009).

Although there has been a focus on antioxidant effects of phytochemicals for many years, it is also recognised that non-antioxidant effects such as effects on cell signalling and gene expression are also important for health (Brahmbhatt, Gundala, Asif, Shamsi, & Aneja, 2013; Poulose, Miller, & Shukitt-Hale, 2014).

3.1.2 The main classes of polyphenolic compounds

Phenolic compounds comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols. Polyphenols (Fig. 3.1) are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. The main groups of polyphenol are: flavonoids, phenolic acids, tannins (hydrolysable and condensed), stilbenes and lignans (Khan & Dangles, 2014).

3.1.2.1 Flavonoids

More than 8000 polyphenolics, including over 4000 flavonoids have been identified, and the number is still growing (Harborne, J. B., Baxter, H., & Moss, 1999). Flavonoids can be further classified into anthocyanins, flavones, isoflavones, flavanones, flavonols and flavanols (Tsao & Yang, 2003). The chemical structures of the main classes of flavonoids are presented in Fig. 3.2.

Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6–C3–C6 configuration. Essentially the structure consists of two aromatic rings, A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C. The aromatic ring A is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway (Merken & Beecher, 2000).

Variations in the substitution patterns of ring C result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (Hollman & Katan, 1999), of which flavones and flavonols are the most widely occurring and structurally diverse (Harborne, J. B., Baxter, H., & Moss, 1999). Substitutions in rings A and B give rise to different compounds within each class of flavonoids. These substitutions may include oxygenation, alkylation, glycosylation, acylation, and sulphonation (Pietta, 2000).

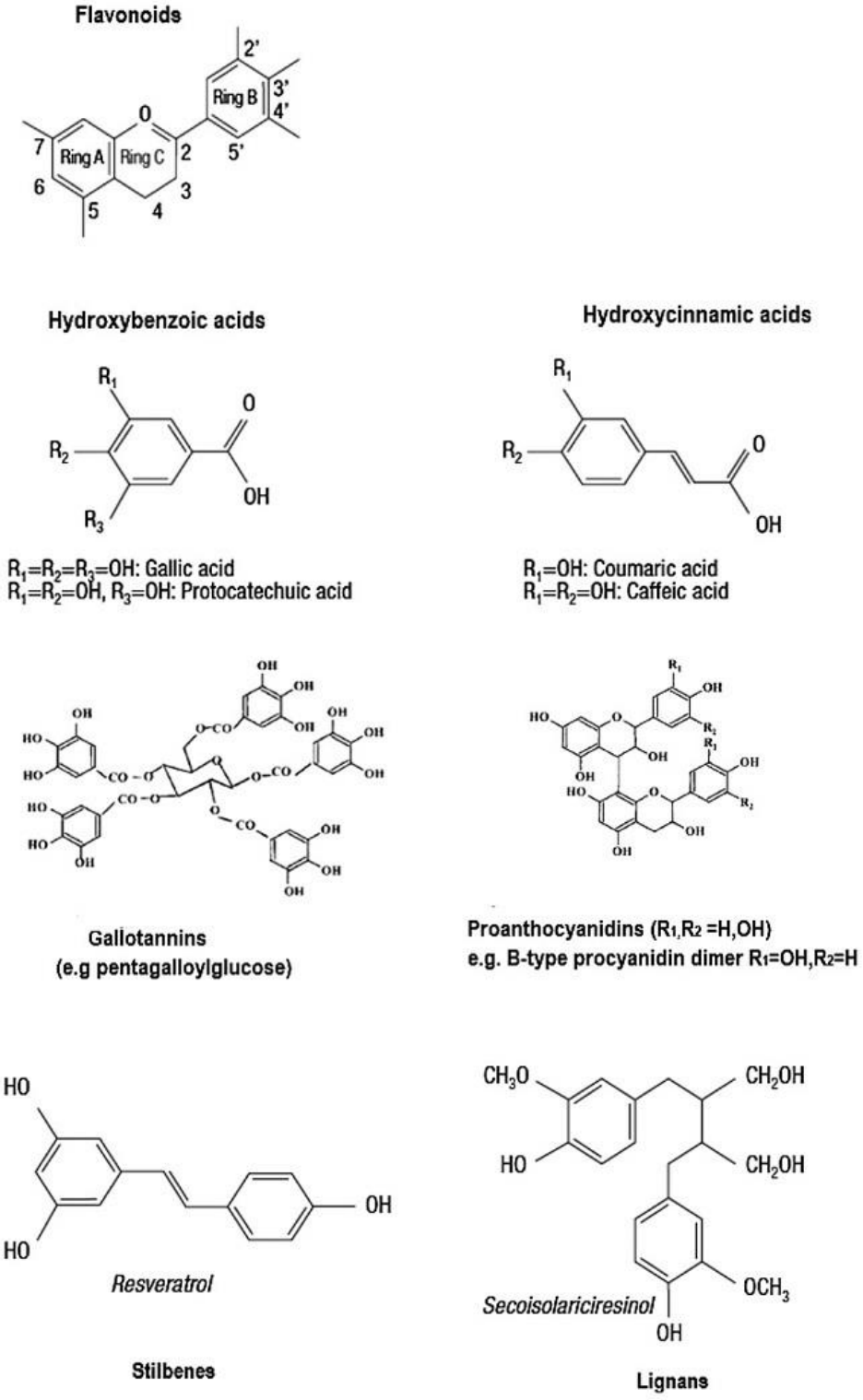

Figure 3.1 Chemical structures of the main classes of phenolic compounds (Ignat et al., 2011).

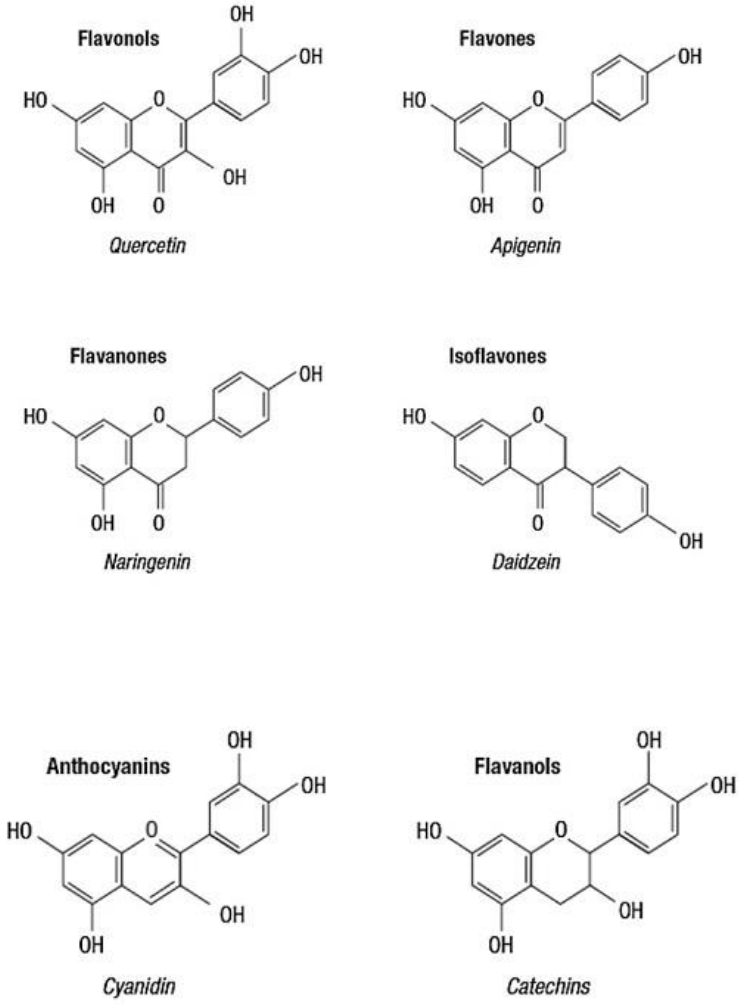

Figure 3.2 Chemical structures of flavonoids (Ignat et al., 2011).

Flavonoids are especially important antioxidants due to their high redox potential, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelating potential (Tsao & Yang, 2003). Flavonoids are the most commonly found phytochemicals, that typically help to protect the plant against UV light, fungal parasites, herbivores, pathogens and oxidative cell injury. When consumed regularly by humans, flavonoids have been associated with a reduction in the incidence of diseases such as prostate (Jaganathan, Vellayappan, Narasimhan, & Supriyanto, 2014; Sharmila et al., 2014) or breast cancer (Yiannakopoulou, 2014). However through *in vivo* experimental studies of anticancer activity of plant flavonoids revealed that there is no clear evidence that dietary flavonoids are associated with reduced risk of stomach and colorectal cancer (Woo & Kim, 2013). It is necessary more preclinical tests and also some

epidemiological studies in order to have a better understanding and promote dietary flavonoids as crusaders against colon cancer.

There is currently great interest in flavonoid research due to the possibility of improved public health through diet, where preventative health care can be promoted through the consumption of fruit and vegetables. Flavonols are a class of flavonoids commonly found in many fruits and vegetables, their content varying widely, depending on environmental factors, such as growing conditions, climate, storage and cooking conditions (Caridi et al., 2007).

Flavanones are characterised by the presence of a saturated three-carbon chain and an oxygen atom at C4. They are generally glycosylated by a disaccharide at C7. Flavanones are present in high concentrations only in citrus fruit, but they are also found in tomatoes and certain aromatic plants such as mint. The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons.

Isoflavones such as estradiol have structural similarities to estrogens, i.e. hydroxyl groups at the C7 and C4, positions. They are phytochemicals that are found in many plants and plant-derived foods in both native ("aglycon") form and as acetyl-, or malony-substituted, β-glucosides. Important health effects are attributed to them, and so it has been suggested that they should be used for the prevention or cure of prevalent diseases such as atherosclerosis or cancer. Some physiological effects are attributed to their structural similarities to β-estradiol, and they are occasionally referred to as "phytoestrogens" (Klejdus et al., 2007).

Anthocyanins are water-soluble vacuolar pigments that may appear as red, purple, or blue depending on pH. They belong to a parent class of molecules called flavonoids, synthesised via the phenylpropanoid pathway. Anthocyanins occur in all plant tissues, including leaves, stems, roots, flowers, and fruits. The anthocyanidins are the basic structures of the anthocyanins. The anthocyanidins (or aglycons) consist of an aromatic ring A bonded to a heterocyclic ring C that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring B. When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins (Konczak & Zhang, 2004).

The glycoside derivatives of the three non-methylated anthocyanidins (pelargonidin-Pg, cyanidin-Cy, delphinidin-Dp) are the most common in nature, being found in 80% of

pigmented leaves, 69% of fruits and 50% of flowers. Six anthocyanidins occur most frequently in plants: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. The sugars commonly linked to anthocyanidins are monosaccharides (glucose, galactose, rhamnose and arabinose), and di- or tri-saccharides formed by combination of the four monosaccharides (Bureau, Renard, Reich, Ginies, & Audergon, 2009). Moreover many anthocyanins have sugar residues acylated with aromatic or aliphatic acids. The isolated anthocyanins are highly unstable and very susceptible to degradation (Giusti & Wrolstad, 2003). Their stability is affected by several factors such as pH, storage temperature, chemical structure, concentration, light, oxygen, solvents, the presence of enzymes, flavonoids, proteins and metallic ions (Castañeda-Ovando et al., 2009). Anthocyanins, as well as other phenolics, can act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation (Iversen, 1999). Their antioxidant potential is dependent on the number and arrangement of the hydroxyl groups and the extent of structural conjugation, as well as the presence of electron-donating and electron-withdrawing substituents in the ring structure (Lapornik, Prošek, & Golc Wondra, 2005). Anthocyanins, coloured natural compounds easily obtained from fruits and vegetables, can be considered potential substitutes for the banned food dyes: they have, in fact, bright attractive colours, while the high water solubility of these compounds allows their easy incorporation into aqueous food systems (Kammerer, Carle, & Schieber, 2004).

3.1.2.2 Phenolic acids

Phenolic acids constitute about one-third of the dietary phenols, which may be present in plants in free and bound forms (Robbins, 2003). Bound-phenolics may be linked to various plant components through ester, ether, or acetal bonds (Zadernowski, Czaplicki, & Naczk, 2009). Phenolic acids consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanilli and syringic acids, which have in common the C6-C1 structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C6-C3), caffeic, ferulic, *p*-coumaric and sinapic acids being the most common representatives (Bravo, 1998).

3.1.2.3 Tannins

Tannins, relatively high molecular compounds which constitute the third important group of phenolics, may be subdivided into hydrolysable and condensed tannins. Proanthocyanidins (condensed tannins) are polymeric flavonoids. Although the biosynthetic pathways for flavonoid synthesis are well understood, the steps leading to condensation and polymerization have not been elucidated. The most widely studied condensed tannins are based on flavan-3-ols: (–)-epicatechin and (+)-catechin (Hagerman, 2012).

Hydrolysable tannins are derivatives of gallic acid (3,4,5 trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins (Hagerman, 2002).

Tannins have diverse effects on biological systems since they are potential metal ion chelators, protein precipitating agents and biological antioxidants. Because of the varied biological roles that tannins can play and because of the enormous structural variation, it has been difficult to develop models that would allow an accurate prediction of their effects in any system. An important goal of future work on the biological activities of tannins is the development of structure/activity relationships so that biological activities can be predicted (Hagerman, 2002).

3.1.2.4 Stilbenes and lignans

Low quantities of stilbenes are present in the human diet, and the main representative is resveratrol, that exists in both *cis* and *trans* isomeric forms, mostly in glycosylated forms (Delmas et al., 2006). It is produced by plants in response to infection by pathogens or to a variety of stress conditions. It has been detected in more than 70 plant species, including grapes, berries and peanuts (Bavaresco, 2003).

Lignans are produced by oxidative dimerization of two phenylpropane units; they are mostly present in nature in the free form, while their glycoside derivatives are only a minor form. The interest in lignans and their synthetic derivatives is growing because of potential application in cancer chemotherapy and various other pharmacological effects (Saleem, Kim, Ali, & Lee, 2005).

3.1.3 Natural Sources of Antioxidants

3.1.3.1 *Caesalpinia spinosa*

Figure 3.3 *Caesalpinia spinosa* (tara) pods (http://www.annalaurent.com, 22.05.2014).

Tara (*C. spinosa*) is a native leguminous tree from South America consisting of red or pale yellow pods of 8-10 cm length. It is spread from the region of Venezuela, Colombia, Ecuador, Peru, Bolivia, until the north of Chile. Tara grows wild in the Peruvian coast and Andean region at altitudes from 1000 to 2900 m above sea level (De la Cruz, 2004). Peru is considered the most important worldwide producer with more than 80% of the world production (Chambi et al., 2013). Tara infusions have been traditionally and extensively used by Peruvian folk medicine to treat inflamed tonsils, fever, cold and stomachaches (Bussmann & Sharon, 2006). Tara pods (without seeds) represent approximately 65% (w/w) of the fruit. Ground tara pods contain a high tannin content (between 40-60% (w/w)), which is of the hydrolysable type, with gallic acid as the main constituent (Aguilar-Galvez et al., 2014). Tara pods are a good source of tannic, gallotannic and gallic acids. Tara tannins are used in the manufacture of leather furniture, as a wine clarifier, and as a source to obtain the antioxidant gallic acid used in the oil industry (De la Cruz, 2004). Anti-inflammatory, antifungal, antibacterial, and antiseptic properties have been attributed to tara tannins (Aguilar-Galvez et al., 2014; Bussmann & Sharon, 2006; Kloucek et al., 2005; Kondo, Takaishi, Shibata, & Higuti, 2006).

3.1.3.2 *Perilla frutescens*

Figure 3.4 Leaves from *Perilla frutescens* (http://www.pamieshorticoles.com, 22.05.2014).

Purple perilla (*P. frutescens*) is a member of the genus of annual herbs belonging to the mint family *Lamiaceae*, which are primarily cultivated in Asian countries such as India, China, Japan, Vietnam, Thailand, Taiwan and Korea. Purple perilla is an edible vegetable used in salads, sushi and soup, as well as for pickles and garnishes (Ha et al., 2012). In general, the stalks, leaves and seeds of perilla are used individually in Chinese medicine to treat a variety of diseases. The stalks of the plant are used as an analgesic and anti-abortive agent. The leaves are said to be helpful for asthma, colds and flus, and to regulate stomach function, while the seeds are employed for dyspnea and cough relief, phlegmelimination, and bowel relaxation (Lin, Chou, Kuo, & Huang, 2010). Red perilla is has strong antioxidant activity and is used as a food colorant owing to the great amount of anthocyanins (Kang & Lee, 2011). Increasingly considerable attention has been given to the antioxidant, anti-inflammatory, anti-allergic and anti-tumor promoting substances contained in perilla (Banno et al., 2004; Makino et al., 2003; Schirrmacher, Skurk, Hauner, & Grassmann, 2010). The main polyphenolic compounds, such as anthocyanidin, luteolin, apigenin, catechin and rosmarinic acid have been isolated and identified from perilla plants (Meng et al., 2006, 2009; Peng, Ye, & Kong, 2005; Yamazaki et al., 2003). It has been reported that the aqueous extract of *P. frutescens* leaves possesses a hepatoprotective capacity against *t*-BHP-induced hepatic and oxidative damage in the rat liver through scavenging reactive oxygen species (ROS) and attenuating the loss of glutathione (GSH) (Kim et al., 2007). Rosmarinic acid, as one of major polyphenolc

compounds in the perilla extract, could reduce lipopolysaccharide-induced and D-galactosamine-induced liver injury (Osakabe et al., 2002). It was reported that luteolin and apigenin function as monoamine transporter activators, which would improve several hypermonaminergic neuropsychological disorders, especially cocaine dependence, through up-regulating monoamine transporter activity (Zhao et al., 2010).

3.1.3.3 *Artemisia annua*

Figure 3.5 Leaves from *Artemisia annua* (http://www.noorherbals.com, 22.05.2014).

Artemisia annua (Asteraceae) has been used throughout the ages in Chinese folk medicine to treat various ailments, specifically those related to the treatment and prevention of fevers which we now relate to malarial infections. Artemisinin, a sesquiterpene lactone, isolated from this herb, was detected as having potent antiplasmodial activity and as being effective against malaria, even in patients with parasites resistant to chloroquine (Wright et al., 2010). However, drugs based on artemisisnin or its semi-synthetic analogues, like other antimalarial drugs, are expensive and not accessible to most people at risk of malaria (Kokwaro, 2009). Therefore, from a practical viewpoint, *A. annua* infusion remains an important source of artemisinin, as a natural antimalarial treatment, and selection strategies to obtain plants or extracts with high levels of artemisinin have been expanded (Bilia, Melillo de Malgalhaes, Bergonzi, & Vincieri, 2006; Ferreira, Luthria, Sasaki, & Heyerick, 2010). Many studies have focused solely on the extraction and quantification of artemisinin without actually conducting a complete chemical profile of *A. annua* formulations. Over the years, more than 600

secondary metabolites have been identified and reported for this herb, including several sesquiterpenoids, triterpenoids, steroids, coumarins, flavonoids, alkaloids, benzenoids, di and mono-terpenoids (de Oliveira et al., 2009; Liu, Zhao, & Wang, 2006; Weathers et al., 2011). As reviewed by Bhakuni et al. (2001) and Ferreira et al. (2010) this compound is reported as having antibacterial, antiviral, anti-inflammatory, plant growth regulatory, cytokine-like and anti-tumor activities.

3.1.3.4 *Viola wittrockiana* Gams.

Figure 3.6 Garden pansies (*V. wittrockiana*) (http://www.pamieshorticoles.com, 22.05.2014).

Viola tricolor (Violaceae), also known as heartsease or wild pansy, has a long history in phytomedicine. Heartsease herb has been utilized to treat various skin disorders, upper-respiratory problems, and also used as a diuretic. Garden pansies (*V. wittrockiana* Gams.) are plants of complex hybrid origin involving at least three species: *V. tricolor, V. altaica* and *V. lutea*. They occur as several colorful varieties and are widely cultivated as ornamental plants. Since garden pansies have been crossbred from *V. tricolor*, they are believed to possess similar good antioxidant activity. Due to its carotenoids, anthocyanins and flavonols content, garden pansies may represent a promising source of natural antioxidants (Gamsjaeger et al., 2011; Vukics, Kery, & Guttman, 2008). Furthermore, edible flowers, such as garden pansies, contribute to the attractive appearance of food. They are used during the cooking preparation, but more often they are mentioned in connection with biologically active substances important for human nutrition (Mlcek & Rop, 2011).

3.1.4 Types of extraction of phenolic compounds

In the last several years, the extraction of phenolic compounds from natural products has attracted a special interest (Pinelo et al., 2005). Extraction is a very important step in the isolation, identification and use of phenolic compounds and there is no single and standard extraction method. Solvent extraction and extraction with supercritical fluid are the most commonly used techniques for the isolation of phenolic compounds (Baydar, Özkan, & Sağdiç, 2004; Bleve et al., 2008).

Phenolic compounds have been extracted by grinding, drying or lyophilizing fruits, vegetables and herbs or simply by soaking fresh plants with subsequent solvent extraction (Merken & Beecher, 2000). These methodologies imply the co-extraction of non-phenolic substances, such as sugars, organic acids and proteins, requiring subsequent purification processes (for example separation by solid phase extraction, SPE). Solvent extraction may be liquid-liquid or solid-liquid extraction depending on the biomass status (Castañeda-Ovando et al., 2009).

3.1.4.1 Liquid – liquid extraction

Liquid-liquid extraction is a mass transfer operation in which a liquid solution (the feed) initially containing one or more solutes is thoroughly mixed with an immiscible or nearly immiscible liquid (solvent). The solvent exhibits preferential affinity or selectivity towards one or more of the components in the feed and has different density. Two streams results from this contact: the extract, which is the solvent rich solution containing the desired extracted solute, and the raffinate, the residual feed solution containing little solute (Ignat et al., 2011).

3.1.4.2 Solid – liquid extraction

Solid-liquid extraction or leaching can be defined as a mass transport phenomenon in which solid contained in a solid matrix migrates into a solvent brought into contact with the matrix. Mass transport phenomena can be enhanced by changes in concentration gradients, diffusion coefficients or boundary layer (Corrales, García, Butz, & Tauscher, 2009). It is a unit operation extensively used to recover many important food components: sucrose in cane or beets, lipids from oilseeds, proteins in oilseed meals, phytochemicals from plants, functional hydrocolloids from algae and polyphenol compounds from plants, fruits, vegetables, etc.

Extraction efficiency is known to be a function of process conditions. Several factors affect the concentration of the desired components in the extract: temperature, liquid–solid ratio, flow rate and particle size (Hayouni et al., 2007; Pinelo et al., 2005). The most common solvents extraction methods are those using acidified methanol or ethanol (Amr & Al-Tamimi, 2007; Caridi et al., 2007). From these methods, extraction with methanol is often the most efficient (Kapasakalidis et al., 2006); in fact, it has been found that in anthocyanin extractions from grape pulp, the extraction with methanol is 20% more effective than that with ethanol, and 73% more effective than water extraction (Castañeda-Ovando et al., 2009; Kapasakalidis, Rastall, & Gordon, 2006). Nevertheless, in food industry ethanol is preferred due to the toxicity of methanol.

Usually, the extraction procedure is sequential and systematically releases the phenolic compounds from the matrix. In the case of phenolic acids (free or bound acids), the first step of the procedure typically involves the use of an aqueous organic solvent to extract soluble/extractable phenolic acids (free, soluble esters, and soluble glycosides) (Russell et al., 2009). Phenolic acids also exist as insoluble bound complexes, which are coupled to cell wall polymers through ester and glycosidic links and are not extractable by organic solvents. Bounds phenolic acids are typically liberated by base hydrolysis, acid hydrolysis or both (Mattila & Kumpulainen, 2002). The main step in most procedures involves base hydrolysis with NaOH ranging from 2 to 10 M, using incubation time up to 16 h, sometimes under nitrogen. Following base hydrolysis, acid hydrolysis is sometimes performed to liberate bound phenolics that have not been previously hydrolysed (Ross, Beta, & Arntfield, 2009).

3.1.4.3 Supercritical fluid extraction

Supercritical fluid extraction (SFE) could be an environmentally beneficial alternative to the conventional organic solvent extraction of biological compounds: SFE methods are rapid, automatable, selective and avoid the use of large amounts of toxic solvents. In addition, the absence of light and air during the extraction reduces the degradation processes that can occur during the traditional extraction techniques (Bleve et al., 2008). SFE is based on the fact that, close to the critical point, the solvent changes its properties rapidly with only slight variations of pressure (Palenzuela et al., 2004).

Supercritical fluids are increasingly replacing organic solvents such as *n*-hexane, dichloromethane, chloroform, and others which are conventionally used in industrial

extraction, purification, and recrystallization operations because of regulatory and environmental pressures on hydrocarbons and ozone-depleting emissions. Supercritical fluids have solvating powers similar to liquid organic solvents, but with higher diffusivity, lower viscosity, and lower surface tension. By far the most utilized critical fluid has been supercritical carbon dioxide (SC-CO_2), due to its benign effect on the environment, low toxicity, non-flammability and compatibility with processed foodstuffs. Furthermore, it has modest critical conditions, it can be readily separated from solutes and it is inexpensive. In natural product extraction and isolation, supercritical fluid extraction, especially that employing supercritical CO_2, has become the method of choice. Sophisticated modern technologies allow precise manipulation of the solvating property of the SCF, which helps the extraction of natural products of a wide range of polarities. By adding modifiers to a SCF (like methanol to CO_2) its polarity can be changed to obtain more selective separation power. Therefore, supercritical carbon dioxide (SC-CO_2) methods are ideal for the extraction of natural products from plant materials and are particularly recommended for the extraction of thermolabile compounds, when low temperatures are required (Nahar & Sarker, 2012).

3.1.4.4 Other extraction methods

Conventional extraction using heating, boiling, or refluxing can be used to extract natural phenolic compounds. However, the disadvantages are the loss of polyphenols due to ionization, hydrolysis and oxidation during extraction, as well as the long extraction time (Li, Chen, & Yao, 2005).

In recent years, various novel extraction techniques have been developed for the extraction of nutraceuticals from plants, including ultrasound-assisted extraction, microwave-assisted extraction and high hydrostatic pressure extraction (L. Wang & Weller, 2006). Amongst these, ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques (Wang et al., 2008). This reported method describes a procedure for extracting non-volatile and semivolatile organic compounds from solids such as soils, sludges and wastes. The ultrasonic process ensures an intimate contact of the sample matrix with the extraction solvent. Ultrasonication is often used to improve the extraction of lipids, proteins and phenolic compounds from plants. Huang et al. (2009) assayed a ultrasound-assisted extraction of

phenolic compounds from *Folium eucommiae,* which was found to be more efficient than extractions by heating, microwave-assisted and enzyme-assisted methods.

Extraction using microwave energy is a largely unexplored area, although by using microwaves to mediate the extraction, it is possible to maintain mild conditions and achieve a superior effect of the extraction. Enzymatic release of phenolic compounds is another useful technique for the extraction of natural polyphenols. High hydrostatic pressure (HHP) is a novel method to enhance mass transport phenomena. Higher caffeine extraction yields from coffee and a higher carotenoid content in tomato puree have been demonstrated when extraction were assisted by high hydrostatic pressure (Sanchez-Moreno et al., 2004).

3.2 Methods for determination of antioxidant activity

In the literature, different antioxidant activity assay methods are employed. Prior et al., (2005) reported that a meeting of the First International Congress on Antioxidant Methods was held in June 2004 at Orlando. Deliberations were made on how to deal with analytical issues relative to assessing antioxidant capacity (AOC) in food, botanicals, nutraceuticals, and other dietary supplements and it was suggested that one or more analytical methods should be standardized for routine assessment of AOC. Standardized test methods will allow for (1) guidance for appropriate application of assays, (2) meaningful comparisons of food or commercial products, (3) a means to control variation within or between products, and (4) provision of quality standards for regulatory issues and health claims. In the standardization, the assay to be employed should be of analytical range, repeatable, possess good recovery, and also be able to recognize interfering substances (Prior et al., 2005).

Huang et al. (2005) and Prior et al. (2005) reported that antioxidant capacity assays can be divided into two different categories based on their chemical reactions or reaction mechanisms which are Hydrogen Atom Transfer (HAT) based assay and Single Electron Transfer (SET) based assay. The end result for these two reactions is the same, but the kinetics and potential for side reactions differ.

HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation (AH = any H donor) to form stable compounds: Eq. 1.

$$X^{\bullet} + AH \longrightarrow XH + A^{\bullet} \quad (1)$$

The HAT-based methods are generally composed of a synthetic free radical generator, an oxidizable molecular probe and an antioxidant (Huang et al., 2005). Most of the HAT-based assays apply a competitive scheme, whereby the antioxidant and substrate compete for peroxyl radicals which are thermally generated through the decomposition of azo-compounds. HAT reactions are solvent and pH independent and usually completed in seconds to minutes (Zulueta, Esteve, & Frígola, 2009).

SET-based methods on the other hand detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Eq. 1-4).

$$X^{\bullet} + AH \longrightarrow H^{-} + AH^{\bullet+} \quad (1)$$

$$AH^{\bullet+} \longleftrightarrow A^{\bullet} + H_3O^{+} \quad (2)$$

$$X^{-} + H_3O^{+} \longrightarrow XH + H_2O \quad (3)$$

$$M(III) + AH \longrightarrow AH^{+} + M(II) \quad (4)$$

The SET-based assay methods involve one redox reaction with the oxidant (also as a probe for monitoring the reaction) as an indicator of the reaction endpoint. These assays analyse the ability of an antioxidant to retain an oxidant which changes colour when reduced. The degree of the colour change is correlated with the sample's antioxidant concentrations (Zulueta et al., 2009). SET reactions are pH dependent, relatively slow and can require a long time to reach completion (Karadag et al., 2009). Compared to HAT, SET is solvent dependent, has the potential to generate new antioxidants through polymerization of phenolic compounds and may underestimate the true antioxidant potential by reactions not reaching their completion.

Different assays utilize different reaction mechanisms some of which are highlighted below (Huang et al., 2005):

Assays that utilize Hydrogen Atom Transfer (HAT):

- ORAC (Oxygen Radical Absorbance Capacity)
- TRAP (Total Radical Trapping Antioxidant Parameter)
- DPPH (Diphenylpicrylhydrazyl)
- IOU (Inhibited Oxygen Uptake)

- Inhibition of linoleic oxidation
- Inhibition of low density lipoprotein (LDL) oxidation

Assays that utilize Single Electron Transfer (SET):

- ABTS (2,2`-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
- FRAP (Ferric Ion Reducing Antioxidant Parametr)
- DPPH
- Copper(II) reduction capacity
- TPC (Total Phenol Content, using Folin-Ciocalteu reagent)

SET and HAT reactions may occur together and the mechanism finally dominating in a system will be determined by the antioxidant characteristics (Prior et al., 2005). The characteristics of the five antioxidant assays ABTS, DPPH, FRAP, ORAC and TPC are considered below:

3.2.1 Folin Ciocalteu Reagent Assay for Total Phenolic Content

The Folin-Ciocalteu assay is used to measure total phenolics by an oxidation/reduction (redox) reaction (Prior et al., 2005). The principle is based on the transfer of single electrons (SET) in alkaline medium from phenolic compounds to molybdenum to form a blue complex that can be monitored spectrophotometrically at 750-765 nm (Magalhães, Segundo, Reis, & Lima, 2008).

Advantages:

- Convenient, simple, precise and reproducible (Huang et al., 2005; Prior et al., 2005).
- Excellent linear correlation with other assays such as: DPPH, FRAP, TEAC, ORAC (Gallego et al., 2013; Karadag et al., 2009).
- The long wavelength of the chromophore minimizes possible interference from the sample matrix, which is often coloured.
- Commonly accepted assay, routinely practiced in research laboratories and hence there is a large body of comparative data (Huang et al., 2005).
- Characterising and standardizing botanical samples.

Disadvantages:

- Suffers from interference from sugar, aromatic amines, sulphur acids, Fe^{2+}.

- Several non-phenolic organic and some inorganic substances can give false values.
- Carried out in aqueous phase and is not applicable for lipophilic antioxidants.
- Standards with more than one reacting OH group give high absorbance backgrounds (Karadag et al., 2009).

3.2.2 ABTS radical cation decolourization assay

The generation of a highly stable chromophoric cation radical of ABTS (blue/green) by peroxyl radicals or other oxidants in the presence of H_2O_2 can be reduced by antioxidants. The antioxidant can delay or diminish its absorbance. In later modifications, reduction of ABTS which relies on electron transfer in monitored (Floegel et al., 2011; Paixao et al., 2007).

The ABTS assay was first proposed by Miller et al. (1996), but it was subsequently improved by Re et al. (1999). The difference between the two methods is the way in which the ABTS is generated. The original ABTS assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants. This technique was criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical (Re et al., 1999). The improved technique for the generation of ABTS involves the pre-formation of the radical cation prior to the addition of the antioxidant.

Advantages:

- An easy and rapid method that produces very reliable results (Paixao, 2007).
- $ABTS^{\bullet+}$ can be solubilized in both aqueous and organic media and is not affected by ionic strength. It can be used to measure antioxidant activity of hydrophilic and lipophilic antioxidants (Arnao, 2000; Karadag et al., 2009).
- Reacts quickly with antioxidants within 30 minutes, can be used over a wide pH range and can be automated for microplate use.

Disadvantages:

- TEAC values characterize the ability of a sample to react with $ABTS^{\bullet+}$ rather than to inhibit the oxidative process.
- The reaction between ABTS and samples may take a long time to reach an end point. An assay with a fixed short time (4-6 minutes) is too short and may give

incorrect antioxidant capacity values because the reaction is incomplete. TEAC values obtained at a fixed end point or measured based on the kinetic behaviour of the samples produce difference results (Huang et al., 2005; Karadag et al., 2009).

- Requires special preparation in which $ABTS^{\bullet+}$ must be generated by enzymes or chemical reaction (Arnao, 2000, Wojdylo et al., 2007).
- The $ABTS^{\bullet+}$ used in TEAC is an artificial radical and is not found in a biological system. Consequently, the assay does not reproduce the *in vivo* situation.

3.2.3 DPPH radical scavenging activity assay

In the presence of a hydrogen/electron donor (free radical scavenging antioxidant) the absorption intensity is decreased and the radical solution (the purple chromogen of DPPH radicals) is discoloured to a pale yellow hydrazine according to the number of electrons captured (Locatelli et al., 2009). DPPH works in both electron transfer (SET) and hydrogen transfer (HAT) systems and allows the determination of a substance or a complex mixture that donate either hydrogen atoms or electrons in a homogeneous system (Paixao et al., 2007).

Advantages:

- A rapid, simple and inexpensive method for estimating the antiradical activity of foods (Paixao et al., 2007).
- It produces stable organic nitrogen radicals characterized by a deep purple colour in the range 515-520 nm (Locatelli et al., 2009).

Disadvantages:

- Some antioxidants such as carotenoids have spectra that overlap with DPPH at 515 nm and interfere with the results (Prior et al., 2005; Karadag et al., 2009).
- DPPH is discoloured from radical reactions (HAT) or reductions (SET) and consequently unrelated reactions may give inaccurate results.
- DPPH radical can only be dissolved in organic solvents (methanol, ethanol, acetone), which is a limitation when interpreting the role of hydrophilic antioxidants (Arnao, 2000; Karadag et al., 2009).
- Several factors may affect the assay such as solvent, pH, sample concentration and reaction time.

- The absorbance of DPPH radical at 515-520 nm after the reaction with an oxidant is reduced by light, oxygen and solvent types (Apak, Güçlü, Ozyürek, & Karademir, 2004; Karadag et al., 2009; Ozcelik, Lee, & Min, 2003).
- Antioxidants that react quickly with peroxyl radicals *in vivo* may react slowly or even be inert to DPPH due to steric effects preventing accessibility.
- Some researchers have indicated a non-linear relationship exists between the antioxidant concentration and the DPPH radical scavenging activity (Eklund-Jonsson, Sandberg, & Larsson Alminger, 2006; R L Prior et al., 2005).
- DPPH is a stable nitrogen radical but it does not reproduce the *in vivo* situation.

3.2.4 Ferric Ion Reducing Antioxidant Power (FRAP) assay

The FRAP assay is based on the ability to reduce yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by electron-donating antioxidants in an acidic medium (Benzie & Strain, 1999; Wojdylo, Oszmianski, & Czemerys, 2007).

Advantages:

- Simple, rapid, inexpensive and robust assay requiring no specialized equipment and can be performed manually or automatically.
- It is totally electron transfer rather than a mixed SET and HAT. In combination with other methods it is a very useful assay to distinguish dominant mechanisms of different antioxidants.

Disadvantages:

- Requires a longer reaction time to detect some polyphenols that react slowly. The order of reactivity of many different antioxidants can vary considerably. Pulido et al. (2000) reported that there are many samples that show these lengthy reaction times, which include: caffeic acid, tannic acid, ferulic acid, ascorbic acid and quercetin.
- Fe^{2+} is a well-known "pro-oxidant" that can react with H_2O_2 to produce a hydroxyl radical (OH^-). This is the most harmful free radical found *in vivo* (Karadag et al., 2009).
- Some antioxidants such as ascorbic acid and uric acid can reduce both Fe^{3+} and reactive species in the FRAP assay so their ability to reduce Fe^{3+} may reflect their ability in reducing reactive species.

- Determines the total reducing power of samples but not all the reductants that reduce Fe^{3+} are antioxidants (Karadag et al., 2009; Nilsson et al., 2005).
- Some antioxidants such as glutathione (GSH) an important antioxidant *in vivo,* can effectively reduce prooxidants but are not able to reduce Fe^{3+} (Karadag et al., 2009).

3.2.5 Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC method is based on the inhibition of the peroxyl-radical-induced oxidation initiated by the thermal decomposition of azo-compounds such as 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) (Prior et al., 2005). The ORAC method uses the fluorescence of B- or R-phycoerythrin (a fluorescent protein) or d fluorescein (a synthetic non protein molecule) as a fluorescent probe. The loss of fluorescence of the probe is an indication of the damage from the peroxyl radical. ORAC measures antioxidant inhibition of peroxyl radical induced oxidation and reflects the classical radical chain breaking antioxidant activity by H-atom transfer (HAT) (Ou et al., 2001; Karadag et al., 2009).

There is some variation in the literature regarding what fluorescent probe should be used. The original assay, as developed by Glazer's laboratory (Delange and Glazer, 1989), utilized B-Phycoerythrin (B-PE) as the fluorescent probe. This probe has been used throughout the literature but has come under considerable scrutiny, especially from Ou et al. (2001). These authors demonstrated that fluorescin (FL) is a superior fluorescent probe when compared with B-PE when conducting the ORAC assay. This is futher supported by a report published by the U.S. Department of Agriculture (2007) in which the ORAC values of 277 foods are listed, and the standard method adopted is that of Prior et al. (2003) in which FL is used, rather than B-PE, as the fluorescent probe.

Advantages:

- Utilises a biologically relevant source of radicals and it is the only method that combines both inhibition time and degree of inhibition into a single quantity (Prior et al. 2003; Thaipong et al., 2006).
- Has recently been adapted to use fluorescein as the fluorescent probe for high-throughput assays.
- Has largely been applied as a method of choice to quantify antioxidant capacity usually in combination with a total phenol content assay.

- Has been applied to measure the antioxidant capacity of botanical and biological samples (Prior & Cao, 2000).
- Can be applied to measure the antioxidant capacity of both lipophilic and hydrophilic components separately using the same peroxyl free radical source and can be used for antioxidants that exhibit distinct lag phases and those that have no lag phases. It is useful for samples that contain multiple ingredients and have complex reaction kinetics.
- Has the ability to use different free radical generators or oxidants, and can measure many different compounds such as antioxidants against peroxyl and hydroxyl radicals.

Disadvantages:

- Can only measure the antioxidant capacity against peroxyl and hydroxyl radicals and not against all reactive oxygen species (e.g. superoxides and singlet oxygen) (Apak et al., 2004; Karadag et al., 2009).
- The substrate (probe) concentration is often smaller than the antioxidant concentration. However, in food systems, the antioxidant concentration is much smaller than the substrate (e.g. lipid). Therefore, the antioxidant capacity measured in a real food system may be incorrect.
- The use of B-PE as a fluorescent probe has limitations such as large inter batch differences, photo bleaching of B-PE after exposure to the excitation radiation, and interaction with polyphenols by nonspecific protein binding. All these factors cause inconsistency in the assay results and false low readings. This limitation can be solved by FL but FL is pH sensitive and must be carefully monitored. FL is not sufficiently lipid soluble and its fluorescence intensity in a non-polar organic solvent is low (Karadag et al., 2009; MacDonald-Wicks, Wood, & Garg, 2006).

In summary, although each antioxidant assay has its own advantages and disadvantages, it is clear that no one antioxidant assay will reflect the total antioxidant capacity of a particular sample. Therefore, it is impossible to use only one assay to evaluate the antioxidant capacity of a plant product. To evaluate antioxidant capacity of a sample it is, therefore, essential to use several assays. However, the selection of assays should be carefully considered in terms of their reliability and consistency. To achieve this, the antioxidant assays should be applicable to both lipophilic and hydrophilic antioxidants

(Karadag et al., 2009). Recently, several reports have suggested that despite the similarity and strong correlations between assays, the results from the different assays and the correlations between these assays have showed inconsistencies (Awika et al., 2003; Ou et al., 2002; Thaipong et al., 2006). In addition, the same antioxidant assay can give widely different results for the same food product when analysed by different laboratories. Therefore, further development and characterization of the assays and the correlations between the assays for a particular food are needed.

3.3 Lipid oxidation in model food systems

3.3.1 Food Lipids

The fatty acids in the lipids of food tissues may be saturated or unsaturated and may be part of the neutral triglyceride fraction or part of the phospholipid fraction. Free fatty acids are electron-deficient at the oxygen atom of the carbonyl group (C=O); unsaturated fatty acids are also electron-deficient at points of carbon-carbon unsaturation (C=C). These electron-deficient regions make fatty acids susceptible to attack by a variety of oxidizing and high-energy agents generating free radicals (Nawar 1996). Triglycerides contain straight chains of primarily 16- to 18-carbon fatty acids and minimal amounts of unsaturated fatty acids. Phospholipids in tissue membranes contain up to 15 times the amount of unsaturated fatty acids (C18:4, C20:4, C20:5, C22:5, and C22:6) found in triglyceridesof animal fats. They are much more susceptible to oxidation because of the increase in the number of points of carbon-carbon unsaturation (C=C) (Brewer, 2011; Elmore, Mottram, Enser, & Wood, 1999).

3.3.2 Lipid Oxidation Mechanism

Traditionally, lipid oxidation is assumed to be an autocatalytic “free radical chain reaction”. However, food products often contain prooxidants that can initiate lipid oxidation reactions, such as transition metals (e.g., iron and copper), photosensitizers and enzymes (e.g., lipoxygenases). In addition, food products are often exposed to harsh environmental conditions that can initiate lipid oxidation reactions such as thermal processing or exposure to UV light. Lipid oxidation involves a complex series of chemical reactions that can be conveniently divided into three stages (Decker & McClements, 2008; Frankel, 1998).

- Initiation – formation of free radicals,
- Propagation – free-radical chain reactions,
- Termination – formation of non-radical products.

The classical lipid oxidation pathway can be described by the following reaction scheme (Decker and McClements, 2008; Frankel, 1998):

Initiation	$LH + In^{\bullet} \rightarrow L^{\bullet} + InH$	(1)
Propagation	$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$	(2)
	$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$	(3)
Termination	$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$	(4)
	$LOO^{\bullet} + L^{\bullet} \rightarrow LOOL$	(5)
	$L^{\bullet} + L^{\bullet} \rightarrow LL$	(6)

3.3.2.1 Initiation

According to Equation (1), the initiation step begins with the abstraction of hydrogen from a fatty acid molecule to form a free alkyl radical ($L^{\bullet}$), which is normally considered to be the "rate-limiting step" in lipid oxidation. This reaction is endothermic and usually occurs very slowly in the absence of initiators (e.g., heat, metal, ions, free radicals, reactive oxygen species, ultraviolet light, etc.) due to its very high activation energy. The initiation of radical formation on a lipid normally occurs at the carbon that requires the least energy to remove the hydrogen atom. The alkyl radical formed is a free radical with an unpaired electron, which is therefore highly unstable.

3.3.2.2 Propagation

The first step of propagation occurs when the alkyl radical ($L^{\bullet}$) formed during the initiation stage interacts with an oxygen biradical to form a peroxyl radical ($LOO^{\bullet}$) (Eq. 2). The peroxyl radical has higher energy than the alkyl radical. Therefore, it is more likely to abstract hydrogen from another unsaturated fatty acid to form lipid hydroperoxide (LOOH) and another alkyl radical (Eq. 3). This stage is referred to as the "self-sustained" radical chain reaction, and it occurs at a high rate and is characterized by a rapid increase of hydroperoxide formation. The lipid hydroperoxides formed are considered to be primary oxidation products. However, once the lipid hydroperoxides are formed, they can decompose which is induced by high temperature during thermal processing or by various types of prooxidants such as transition metals and UV light.

Hydroperoxides themselves do not contribute to an off-flavor, but they are substrates for rancidity due to the fact they are decomposed into low molecular weight volatile compounds (Decker and McClements, 2008). Decomposition of hydroperoxides (LOOH) can involve a "homolytic cleavage" between the two oxygen atoms of the hydroperoxide to form an alkoxyl ($LO^{\bullet}$) and a hydroxyl ($^{\bullet}OH$) radical (Min & Boff, 2002).

Alternatively, reduced transition metals can decompose lipid hydroperoxide in a reaction where an electron is transferred to the lipid hydroperoxide to form an alkoxyl radical ($LO^{\bullet}$) and a hydroxyl anion (^{-}OH). The alkoxyl radical ($LO^{\bullet}$) is more energetic than the alkyl ($L^{\bullet}$) or peroxyl ($LOO^{\bullet}$) radicals, therefore, they can abstract a hydrogen from another unsaturated fatty acid to further propagate the reaction, attack a pentadiene group within the same fatty acid which can produce cyclic compounds, or abstract an electron from the covalent bonds adjacent to the alkoxyl radicals to cleave the fatty acid chain.

3.3.2.3 Termination

Termination steps occur when two free radicals interact turning into nonradical oxidation products. The end products can vary depending on the type of radicals interacting together (Eqs. 4, 5, 6). Most of the time, the food is already rancid before termination reactions are highly prevalent. An exception can be frying oils where low oxygen concentrations favour termination reactions versus lipid hydroperoxide formation and decomposition.

3.3.3 Kinetics and products of lipid oxidation

In lipid oxidation the process generally show a lag phase followed by an exponential increase in oxidation rate. During the lag phase the oxidation is relatively slow at a steady rate. Increasing the length of this phase as much as possible by lowering temperature, reducing oxygen concentration, reducing activity of prooxidants and increasing concentration of antioxidants, is important from a quality perspective as there are no decomposition products formed and hence no related rancidity in this phase. Once the exponential phase is reached, fatty acid decomposition products quickly form (Fennema, O.R., Parkin, K.L. and Srinivasan, 2007).

Hydroperoxides are the main primary oxidation products, accumulating during the initiation and propagation step of the oxidation process. The time to reach maximum level of hydroperoxides in the oxidation process is related to degree of saturation, and occurs

earliest in highly unsaturated lipids because their hydroperoxides decompose more easily. After the maximum hydroperoxide level has been reached, a drop in hydroperoxides will theoretically be seen as the hydroperoxides decompose into a variety of secondary products (Frankel, 2005).

The drop in hydroperoxide concentration is observed when the rate of decomposition into secondary products exceeds the formation rate. In theory this means that the primary oxidation products will dominate in the early stage and secondary oxidation products will dominate in later stages of the oxidation process (Fig. 3.7).

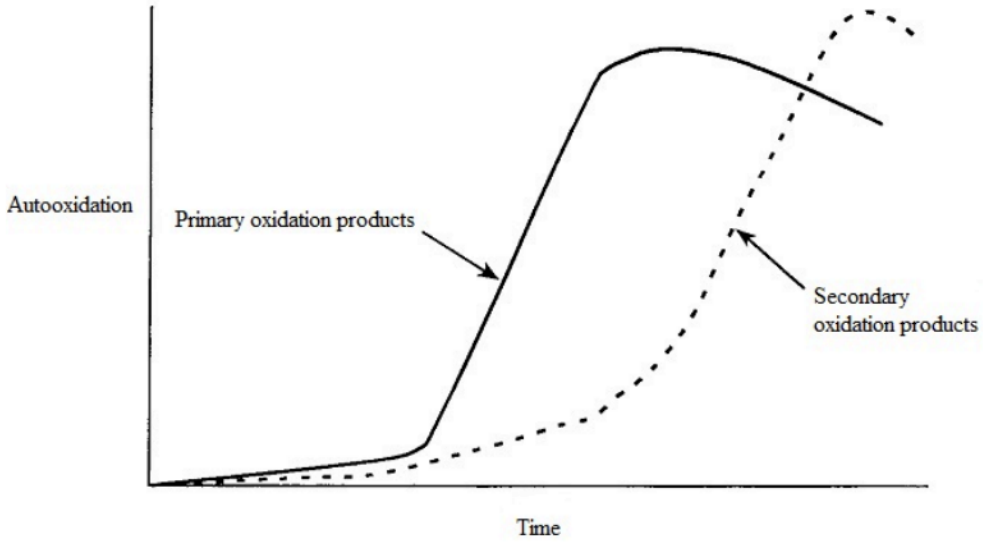

Figure 3.7 Theoretical development of primary and secondary oxidation products as a function of time in lipid oxidation (Frankel, 2005).

3.3.4 Evaluation of the ability to inhibit lipid oxidation in model systems

In contrast to the radical scavenging assays, the assay based on model systems involves relevant oxidation substrates. The evaluation of antioxidants in model systems should be based on measuring changes in the concentration of compounds being oxidized, on depletion of oxygen or on formation of oxidation products. The most commonly used markers are presented in Fig. 3.8.

Several methods are available to measure the same or different indicators of the progress of lipid oxidation. The researcher needs to define the measurement strategy and select the indicators to follow depending on the purpose of the research especially since lipid oxidation is a multistep reaction with a wide range of products and product groups. Conventional measurements include assessment of total hydroperoxide levels and a selection of indicators of secondary oxidation (hydroperoxide decomposition) products

usually by simple titrimetric or spectroscopic techniques. Chromatographic techniques (gas chromatography and/or high performance liquid chromatography together with different detectors including mass spectrometry) are useful for more detailed description of the reaction products (Decker, Warner, Richards, & Shahidi, 2005).

The concentration of primary and secondary products can be measured quantitatively and therefore give an indications of the oxidative status of the oil or fat. Peroxide Value (PV) and Thiobarbituric Acid Reactive Substance (TBARs) test are the two most frequently used quality parameters, measuring primary and secondary oxidation products respectively (Sun, Wang, Chen, & Li, 2011).

Peroxide value directly measures the concentration of hydroperoxides formed in the initial stage of lipid oxidation. The American Oil Chemists' Society has several official methods available in a variety of versions (Sun et al., 2011). In this thesis PV was measured by the spectrophotometric ferric thiocyanate method.

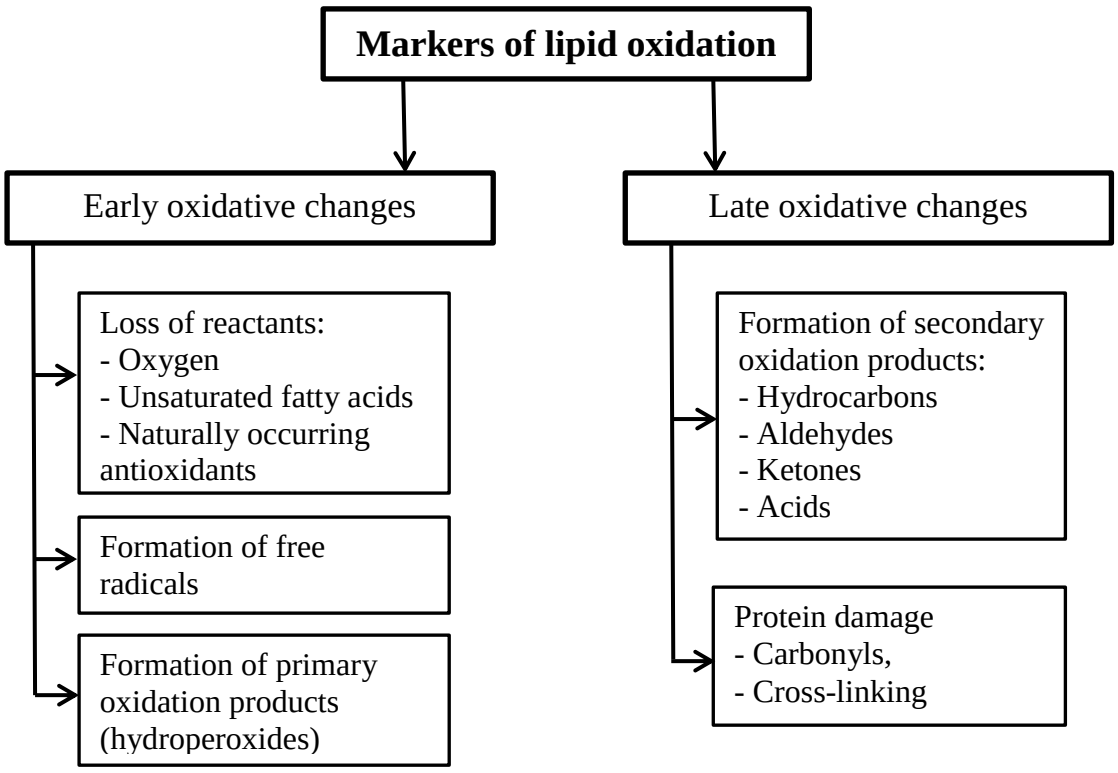

Figure 3.8 Markers of oxidative changes in lipid model systems (Becker, 2004).

The ferric thiocyanate method is based on the ability of hydroperoxides to oxidize ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) in an acidic medium. The ferric ions form chromophores when complexed to thiocyanate, which can be measured by spectrophotometry (Eymard

& Genot, 2003). Ferric thicyanate is a red-violet complex with absorption at 500-510 nm. This method for PV determination in edible oils is simple, reproducible and sensitive (>0.05 mEq peroxide/kg).

A large variety of secondary oxidation products are produced through decomposition of fatty acid hydroperoxides. Small quantities and the large variations in chemical structure and properties, make it difficult to measure all the compounds simultaneously. Analysis for determination of secondary oxidation products therefore tends to focus on a single compound or group of compounds. One drawback of secondary oxidation product measurements is the lack of detection at an early stage in the oxidation process where primary products are high, while secondary ones are low. In many food systems the appearance of amine and sulfhydryl groups also make the measurement of e.g. aldehydes difficult, as they react with secondary oxidation products and thereby lower their concentration. Some authors argue that an advantage of measurement of secondary oxidation products is the good correlation with sensory analysis, as the measured compounds, if volatile, are the direct cause of the off-aromas (Fennema et al., 2007).

The TBARs assay is one of the oldest methods used for detection of lipid oxidation. The method is based on the formation of a pink complex with strong absorbance at 532-535 nm when thiobarbituric acid (TBA) and oxidation products from unsaturated fatty acids react. The reacting secondary products are generally referred to as TBA-reacting substances (TBARs), because the reaction can involve several secondary oxidation products. Initially the reaction was believed to involve a reaction between 2 moles of TBA and one mole of malonaldehyde, as illustrated in Fig. 3.9. Based on this, the test is still standardized by using malonaldehyde generated from 1,1,3,3-tetraethoxyprpane by acid hydrolysis (Frankel, 2005).

The TBARs assay is frequently used in spite of its well-known limitations in lack of sensitivity and specificity. Reaction conditions such as temperature, time of heating, pH, and presence of antioxidants and metal ions are known to significantly affect the colour development (Antolovich et al. 2002). However, the main limitations arise from the ability of several compounds to react with the TBA reagent and hence contribute to an overestimation of the intensity of the colour complex (Sun et al, 2011). Examples of such absorbing compounds are alkanals, 2- alkenals, 2,4-alkdienals, ketones, ketosteroids,

acids, esters, proteins, sucrose, urea, pyridines and pyrimidines (Jardine, Antolovich, Prenzler, & Robards, 2002).

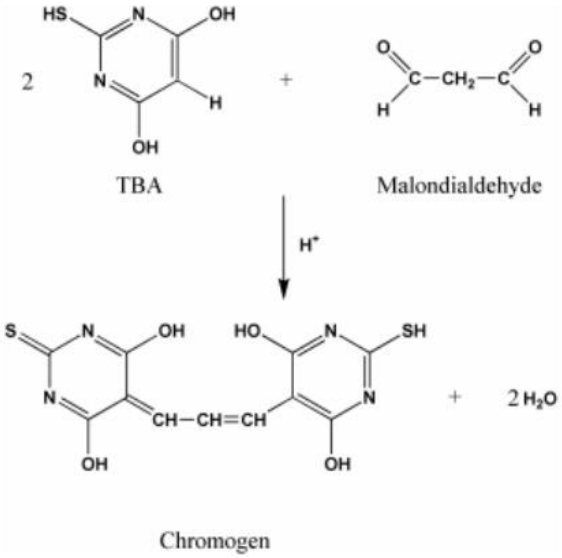

Figure 3.9 The reaction of thiobarbituric acid (TBA) and malonaldehyde (MDA) to form a pink complex, which strongly absorbs in the UV range 532-535 nm (Antolovich et al., 2002).

Lipid oxidation produces various volatile compounds including hydrocarbons, aldehydes, enals, dienals, ketones, and organic acids. As oxidation increases, these volatiles increase and can be measured by injecting a portion of the headspace into a gas chromatograph (GC). In this thesis the hexanal content from lipid oxidation of oil-in-water emulsions with added perilla extracts was monitored during storage by headspace GC.

3.3.5 Emulsion model systems

Many lipid containing processed foods are either water-in-oil emulsions for example, butter and margarine, or oil-in-water emulsions, for example, mayonnaise, milk and cream (Conde, Gordon, Moure, & Dominguez, 2011). While oxidation is a problem in both, the majority of research has been done in oil-in-water system. As well as the food industry, the cosmetics, pharmaceutical and medical industries also utilize oil-in-water emulsions as means to encapsulate, protect and release bioactive lipids in their products. For this reason, the number of studies attempting to understand the physicochemical mechanisms underlying lipid oxidation in oil-in-water emulsions has increased dramatically during the past decade.

Oxidation reactions occur due to the interaction of oxygen with unsaturated fatty acyl groups in lipids. The rate of lipid oxidation in a particular product depends on a number of factors: the presence of oxygen which is required for the development of oxidative rancidity; the chemical composition of the lipids (polyunsaturated fatty acids are more susceptible than monounsaturated fatty acids); temperature (oxidation usually occurs more rapidly at higher temperatures except in some conditions where high temperature limit oxygen solubility); the presence of prooxidants; the nature of the reaction environment (Kim and Min, 2008).

Lipid oxidation is a great concern in the food industry because it causes physical and chemical deterioration, such as losses in important nutrients, formation of potentially toxic reaction products (such as aldehydes and ketones), undesirable changes in appearance and texture, and development of rancidity that shortens product shelf life (Decker & McClements, 2008; Frankel, 1998). Lipid oxidation is favoured in oil-in-water emulsions because of the large contact surface between the oxidizable lipid droplets and water-soluble compounds including oxygen and pro-oxidants, which contribute to the initiation and propagation of oxidation reactions (Frankel, 1998; Villiere et al., 2005).

Lipid oxidation in emulsions is generally recognised as being more complex than lipid oxidation in bulk oil systems, since the emulsification process will lead to the formation of a large interfacial area, and lipid oxidation is initiated at the interface between oil and water, where different non-polar and polar compounds in the system can interact. The efficacy of antioxidants in bulk oil and in dispersed systems is affected by their polarity as described by the "polar paradox". According to the polar paradox, polar antioxidants like ascorbic acid and Trolox are more active in non-polar media, like bulk oils, than less polar components including ascorbyl palmitate and tocopherol (Conde et al., 2011).

Although major differences will exist in the behaviour of antioxidants in different kinds of foods, there are some general considerations that should be observed for all food products (Decker et al., 2005):

- Avoid high oxidation temperatures (>60 °C) during storage studies because the mechanisms and kinetics of oxidation are not the same as at lower temperatures.
- Ensure that the starting lipid does not contain high levels of oxidation products (e.g., hexanal, 2,4-decadiental).

- Effectively analyze the activity of antioxidants by measuring both primary (e.g., hydroperoxides, conjugated dienes) and secondary oxidation (carbonyls, volatile compounds) products.
- Types of fatty acid decomposition products formed during oxidation are related to the fatty acid composition of the oil.
- Use either crude extracts of biological materials or pure compounds as the source of antioxidants. If phenolic compounds are expected to be the major antioxidants in a crude extract, the total phenol content and compositional data of the extract should be reported in order to compare samples. Include a reference compound in the study such as BHA, BHT, TBHQ or one of the tocopherol homologues.
- pH can affect oxidative reactions by influencing pro-oxidant (e.g., iron solubility increases with decreasing pH) and antioxidant (the pH can alter the charge of antioxidants, which can affect solubility and chelation capacity) activity. The pH of oxidation models should therefore be similar to the food of interest.
- Standardize time and conditions to determine if the antioxidant is effective.

An emulsion consists of two immiscible liquids (usually oil and water), with one liquid being dispersed as small (diameter = 0.1 – 100 μm) spherical droplets in the other liquid. Food emulsions can exist as oil-in-water emulsions where oil is dispersed in water or water-in-oil emulsions where water is dispersed in oil. Emulsions are thermodynamically unstable because of the positive free energy needed to increase the surface area between oil and water phases (McClements, 1999). For this reason emulsions tend to separate into a layer of oil (lower density) on top of a layer of water (higher density) with time. To form emulsions that are kinetically stable for a reasonable period (a few weeks, months), chemical substances known as emulsifiers must be added prior to homogenization. The most common emulsifiers used in the food industry are surface-active proteins (e.g., casein, whey, soy and egg) and small molecule surfactants (e.g., Tweens, fatty acids).

3.3.6 Meat model systems

Muscle tissue is composed of a multitude of endogenous components that can either accelerate (prooxidants) or inhibit (antioxidants) oxidative processes. The concentrations and activity of pro-oxidants and antioxidants in muscle foods can change dramatically during processing and storage (ascorbate, glutathione and tocopherol decrease in concentration with increased storage time) (Decker et al., 2005). The main heme proteins

in muscle tissue are haemoglobin (Hb) and myoglobin (Mb) Heme proteins dictate the appearance (e.g., colour) of muscle foods. In addition, Hb and Mb have the capacity to promote lipid oxidation during storage which leads to off-odours and off-flavors. Immediately after death, Mb and Hb are mostly in their "reduced" forms in which the iron atom of the heme group is in the +2 (ferrous) oxidation state. This provides red pigments to the muscle that are often desirable. With increased storage time, the iron atom in the heme ring becomes oxidized to the +3 (ferric) oxidation state resulting in a brown colour which is often undesirable. The oxidized pigments are termed metHb and met Mb. The process of met formation is termed "autooxidation". Only reduced Hb and Mb can bind and release O_2. Sodium nitrite and reductants are used to "cure" processed muscle foods (Nunez de Gonzalez et al., 2008). The nitric oxide (NO) produced during curing binds to the ferrous iron atom in the heme ring in Hb and Mb which results in a pink appearance after thermal processing. Thermal processing in the light and traces of O_2 cause NO-heme pigment to become oxidized resulting in brown pigments. Thus, Hb and Mb are responsible for both desirable and undesirable colour attributes in muscle foods.

Lipid oxidation causes the formation of off-odours and off-flavours (e.g., rancidity) during storage of raw muscle. The term warmed over flavour (WOF) is used to describe off-flavour due to lipid oxidation that develops during storage of cooked meat. Physical changes also occur with increased storage time and processing steps, which bring previously segregated reactants together. Minced trout muscle was considered to be susceptible to rancidity due to the dispersion of blood pigments in the flesh caused by the mechanical destruction of the tissue. Cooking will cause denaturation of proteins, increase the concentration of low molecular weight metals and alter lipid phases. These changes may cause an antioxidant that is effective in the raw state to have a different efficacy in the cooked state (Brewer, 2009; Karre, Lopez, & Getty, 2013; Lara et al., 2011).

Factors to consider when antioxidant efficacy and the mechanisms by which antioxidants inhibit lipid oxidation in meat food systems are assessed include (Decker et al., 2005):

- The oxidative stability of skeletal muscle can show large animal-to-animal variations. Therefore, when antioxidants are assessed in either muscle food or muscle food models, it is best to pool together the muscle from several animals to minimize these types of variations.

- Antioxidant evaluation should be conducted in muscle samples with similar fat contents and the fat concentration should be similar to that of the processed muscle food of interest.
- Partitioning of antioxidants into either the membrane phospholipids or triacylglycerols can also be dependent on how the antioxidant is added to the muscle sample.
- Surface tissue (1-2 mm) should be removed and assayed when intact steaks and fillets are analysed.

Model systems to assess antioxidant effectiveness:

- Raw ground muscle foods
- Cooked ground muscle foods

3.3.7 Natural antioxidants in preservation of food products

For many years, synthetic antioxidants such as BHA and BHT have been considered practical and effective inhibitors of lipid oxidation but recently there has been a trend to replace them with natural antioxidants due to the possible toxicity of synthetic antioxidants to consumers (McBride, Hogan, & Kerry, 2007). These synthetic antioxidants have been restricted by legislation because they are suspected of having some possible carcinogenic affects (Hirose et al., 1998). Consequently, research has concentrated on the efficacy of including natural antioxidants in foods (Table 3.1).

It is should be noted that the ability of antioxidants to intercept or remove free radicals can contribute to preventing the deterioration of food products. Ideally, antioxidants should not affect the organoleptic properties of food and should be effective at low concentrations. However, in the past, a major obstacle to use of plant extracts as ingredients has been the undesirable flavours and odours associated with the extracts. Rosemary has been shown to have high antioxidant capacity but some of the compounds in rosemary such as verbenone, borneol and camphor can impart an undesirable rosemary odour to food even at low concentrations (Brewer, 2011). Red cabbage and radish extracts contain anthocyanins and aroma and flavour compounds that give strong vegetative notes to these extracts (Giusti & Wrolstad, 2003). However, technological developments have resolved this problem by producing antioxidant extracts without any sensory characteristics but with antioxidant properties. Such technologies include solvent extraction, hydro-distillation, spray-drying, freeze-drying and supercritical fluid

extraction (McBride et al., 2007). In addition, the limitations of using a plant extract as a natural antioxidant are availability and cost. Conventionally, plants are harvested in a particular season, and hence the costs of plant sources vary, depending on the season. Moreover, synthetic antioxidants, such as BHA and BHT, are cheaper than natural ones (Maisuthisakul & Gordon, 2009). There is, therefore, considerable interest in finding new, safe and inexpensive antioxidants from natural sources.

Table 3.1 Summary of some previous reports on effects of natural antioxidants on inhibition of lipid oxidation in model food emulsions.

Model food system	Antioxidant	Concentration	Storage condition	Methods used to determine oxidation	Reference
Oil-in water (O/W) emulsions	Green tea and rosemary extracts	0.03 and 0.05%	30 days 30°C	PV CD	Roedig-Penman & Gordon (1997)
Algae and linseed O/W emulsions	Melissa officinalis extract	620 ppm	15 days 25 °C	TBARs	Garcia-Iniguez de Ciriano et al., (2010)
30% (v/v) olive oil and soybean O/W emulsions	Eugenia pollicina leaf extract	0.02%	13 days 40°C	PV CD p-anisidine	Ramful et al. (2011)
Sunflower O/W emulsions	Extracts from marigold, paprika and annatto	0.25-1.5 g/L	27 days 60°C	CD TBARs	Dimakou & Oreopoulou (2012)
Linoleic acid emulsion, sunflower O/W emulsion and bulk sunflower oil	Extract of fermented soybeans	0.1, 0.5, 1, 2, 5 and 10 mg/g	24 days 60°C	CD TBARs	Wardhani et al. (2013)
Canola O/W emulsions	Canola extract	100 and 350 µM	14 days 3 ± 1°C	PV Volatile oxidation products	Sorensen et al., (2013)
Sunflower O/W emulsions	Rosemary, thyme and lavender extracts	100 ppm	40 days 33 ± 1°C	PV TBARs pH	Gallego et al., (2013)

Many food sources can be used as natural antioxidants in meat products. Most of the antioxidant food materials that have been tested in meat products are of plant origin (fruit, vegetables, seeds, spices, etc.) (Table 3.2). A number of researchers have attempted to improve the quality of meat by using natural antioxidants such as vitamin C, vitamin E

and extracts from rosemary, oregano, sage, onion and cranberry. Studies have shown that the natural plant antioxidants can scavenge free radicals, inhibit lipid oxidation and extend the shelf life of food products.

However, several of the reports have indicated that the effectiveness of the various antioxidants in a food system depends on factors such as the physical state of the substrate and the solubility, phase partitioning and concentration of the antioxidants (McBride et al., 2007). It is now recognized that suitable carrier systems may be required to direct and aid the incorporation of antioxidants to a specific location in prepared meat products. Furthermore, the synergistic interactions between hydrophilic and lipophilic antioxidants can also have an effect on the efficiency of incorporating antioxidants into meat products.

Table 3.2 Summary of some previous reports on effects of natural antioxidants on extending shelf life of meat products.

Antioxidant	Concentration	Type of meat	Effects on shelf life	References
Rosemary and oregano extracts	0.01%	Raw pork batters	Reduced TBARS and maintained colour	Hernandez-Hernandez et al., (2009)
Sage	0.1%	Minced chicken breast	Reduced lipid oxidation	Mariutti et al., (2011)
Lotus leaf and barley leaf powder	0.1 and 0.5%	Cooked ground pork	Reduced lipid oxidation (POVs, CD and TBARS)	Choe et al., (2011)
Grape seed extract	0.01, 0.03 and 0.05%	Pre-cooked, frozen, re-heated beef sausages	Reduced lipid oxidation	Kulkarni et al. (2011)
Adzuki bean extract	0.2%	Cured and uncured cooked pork sausages	Reduced lipid oxidation	Jayawardana et al., (2011)
Melissa officinalis extract	965 ppm	Bologna-type sausages enriched in omega-3 fatty acids	Reduced TBARS and PV	Berasategi et al., (2011)
Morinda citrifolia	2, 4 and 6%	Beef patties	Reduced TBARS and maintained colour	Tapp et al., (2012)
Broccoli powder extract	1, 1.5 and 2%	Goat nuggets	Reduced TBARS	Banerjee et al., (2012)
Kiam wood extract	0.04% and 0.08%	Fish emulsion sausages	Reduced TBARS and PV	Maqsood et al., (2012)
Brown seaweed extract	0.01, 0.1 and 0.5%	Cooked minced pork patties	Reduced lipid oxidation (0.5%)	Moroney et al., (2013)

3.4 Methods for quantification and possible identification of antioxidant compounds

There is an increasing demand for highly sensitive and selective analytical methods for the determination of polyphenols (Liu, Cai, & Shao, 2008). Despite a great number of investigations, the separation and quantification of different polyphenolics remain difficult, especially the simultaneous determination of polyphenolics of different classes (Tsao & Yang, 2003).

The isolation and identification of phenolic-based plant extracts typically use some form of chromatography. The term chromatography refers to a process in which chemical mixtures are separated into different components based on variations in their interactions with stationary phase (Anderson & Markham, 2006).

Spectrophotometric methods provide very useful qualitative and quantitative information; actually, spectroscopy is the main technique used for the quantification of different classes of polyphenols due to its simplicity and low cost. The main disadvantage of the spectrophotometric assays is that they only give an estimation of the total phenolic content. It does not separate nor does it give quantitative measurement of individual compounds.

3.4.1 Thin Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is still widely used for the purification and isolation of anthocyanins, flavonols, condensed tannins and phenolic acids using different solvent systems (Naczk & Shahidi, 2006). TLC is a technique with large applicability in the fields of plant material analysis and stability tests of extracts and final products. The implementation of a modern standardized methodology led to an increasing acceptance and recognition of TLC as a competitive analytical method. TLC has many advantages, such as lower cost, short analysis time, the possibility of multiple detection, and specific derivatisation on the same plate.

The separation of polyphenols from each other and from other components of the plant extracts can be carried out by a great number of TLC developed techniques. Mostly, complex crude plant extracts are screened for antioxidant activity or for distinguishing the components of plant extracts with antioxidant character or radical-scavenging properties. TLC has been used to determine individual antioxidant capacity of target compounds and

might be of interest for routine chemical or biological screening, the method offering solutions to real analytical problems (Cimpoiu, 2006).

The TLC-DPPH assay can be regarded as one of the popular tests performed for assessing free radical scavenging ability of individual components found in plant extracts. It has been used, for example, in the so-called biologically guided fractionation, which led to isolation of antioxidants present in plant extracts. The popularity of this assay stems from a variety of TLC advantages, namely, flexibility, high sample trough put, direct access to separated components, ability to detect the activity of individual compounds present in complex samples, easy accessibility by both sophisticated and no specialized equipment. As it has been shown recently, the test can be used to obtain quantitative results, as well, giving the possibility to compare compounds with different free radical scavenging activities (Kowalska et al., 2013). Gu et al. (2009) described an activity-guided isolation of natural antioxidants from fruit of Perilla frutescens by TLC bioautography method. Hawryl and Waksmundzka (2013) reported the possibility of using micro-TLC in two-dimensional separations of some medicinal plant extracts to obtain the information about their composition and antioxidative activity using DPPH as spraying reagent for post-chromatography derivatization.

3.4.2 High performance liquid chromatography (HPLC)

Amongst the different methods available, HPLC is preferred for the separation and quantification of polyphenols in plant materials. The chromatographic conditions of the HPLC methods include the use of, almost exclusively, a reversed-phase C18 column; UV-Vis diode array detector and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). Reverse phase HPLC has become a dominating analytical tool for the separation and determination of polyphenols with different detection systems, such as diode array detector (DAD), mass or tandem mass spectrometry (Ignat et al., 2011).

Nevertheless, due to the disadvantages in detection limit and sensitivity, HPLC methods present limitations especially in complex matrices, such as crude plant extracts and environmental samples. Thus, an initial preconcentration and purification of the polyphenols from a complex matrix is crucial prior to instrumental analysis by HPLC. The aim of pre-concentration is to simplify the chromatograms obtained so that components can be reliably identified and quantified. The purification stage is the critical

part of a method, the removal of potential interfering components varies according to the matrix to be analysed. Polyphenols can usually be purified by adsorption-desorption processes by using highly efficient sorbents, of which C18 and highly crosslinked styrene-divinylbenzene (S-DVB) copolymers are very popular (Liu et al., 2008).

Lower molecular mass polyphenols can be analysed by HPLC on reversed-phase or normal phase columns. However, these techniques are time consuming and can have poor resolution as the polymer chain length and structural diversity increase. The detection of higher molecular weight compounds, as well as the determination of molecular mass distributions, remain as major challenges in the analysis of polyphenols (Fulcrand et al., 2008).

3.4.3 Liquid Chromatography – Mass Spectrometry (LC-MS)

Chromatography – Mass Spectrometry techniques are nowadays the best analytical approach to study polyphenols in vegetable samples, and are the most effective tool in the study of the structure of anthocyanins. LC-MS allows the characterization of complex structures such as procyanidins, proanthocyanidins, prodelphinidins, and tannins, and provides experimental evidence for structures that were previously only hypothesized (Flamini, 2003).

This technique combines the sample separation characteristics of HPLC with the ability to analyse the mass of analytes contained within a sample mixture. The use of a mass spectrometer enables the identification of structures which have been separated by an HPLC technique. This method allows for the measurement of compounds in the form of mass-to-charge ratio and results are reported as a mass spectrum. A compound's mass spectrum is based on the distribution of fragment ions generated from the dissociation of a given analyte (Harborne & Williams, 2000). The detection, interpretation and identification of compounds is based on the theory that different structures often have unique molecular weights and in most cases, dissimilar dissociation patterns. Understanding the dissociation pathways of different types of compounds allows researchers to properly interpret the various fragment ions in a mass spectrum, allowing for the identification of a given structure (Liwei Gu et al., 2003).

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4 Antioxidant properties of extracts of tara (*C. spinosa*) pods *in vitro* and in model food emulsions

4.1 Introduction

The tara tree (*Caesalpinia spinosa*), which belongs to the Caesalpinaceae family, is a native species of Peru that is widely distributed in Latin America. Tara is a small tree that is 2 to 3 m in height with flat, oblong indehiscent orange pods that contain 4 to 7 rounded seeds (Romero, Fernandez, & Robert, 2012). Tara seeds are the source of a thickening gum used in several foods and paintings. In addition, tara pods are ground and used as raw material for the extraction of several compounds that have interesting applications in cosmetics, medicine and the chemical and pharmaceutical industry (De la Cruz, 2004). Moreover, its extracts have been reported to have antitumor, antimicrobial and antioxidant activity (Kloucek, Polesny, Svobodova, Vlkova, & Kokoska, 2005; Kondo, Takaishi, Shibata, & Higuti, 2006).

Tara tree was traditionally considered the second tannin feed stock after *Schinopsis balansae* (Sanchez-Martin, Beltran-Heredia, & Gragera-Carvajal, 2011). Tannins are common secondary metabolites in vascular plants, developing functions that concern plant defense from physical damage and/or pathogen attacks. On the basis of their structural characteristics, tannins are classified into two main groups: hydrolysable tannins (HTs) and condensed tannins (CTs). In HTs, a carbohydrate (usually α-glucose) is partially or totally esterified with phenolic molecules such as gallic acid (giving gallotannins) or ellagic acid (giving ellagitannins) (Annalisa Romani, Campo, & Pinelli, 2012). On the other hand, CTs are oligomeric or polymeric flavonoids based on flavan-3-ol units (commonly catechin or epicatechin) linked via carbon–carbon bond (Hagerman, 2012). Although tara pods composition has not been clearly established yet, its beneficial properties have been mainly attributed to the presence of HTs (Castaneda, Miguel Pombo, Patricia Uruena, Fredy Hernandez, & Fiorentino, 2012). Among them, tannic acid is well known for its ability to induce beneficial effects on human health through the expression of some biological activities (Marienfeld, Tadlock, Yamagiwa, & Patel, 2003).

Recent studies have reported other properties of tannins that make them suitable for being used as astringent agent, for eliminating parasites and as antipyretic (Lee, Chen, Liang, & Wang, 2010). In addition, its ability to reduce serum cholesterol and triglycerides, and to

suppress insulin-stimulated lipogenesis has also been documented (Ong, Khoo, & Das, 1995; Yugarani, Tan, & Das, 1993). Meanwhile, other studies revealed that its antioxidant activity seems to be correlated with its copper chelating capacity (Andrade et al., 2005).

In general, it is known that plant extracts containing tannins are able to interact with biological systems through the induction of some of the aforementioned effects. Accordingly, some extracts have recently been distributed commercially in order to take advantage of their properties (Lizarraga et al., 2007; Romani et al., 2006). In addition, such distribution is expected to grow rapidly in the next years due to a fostered interest in searching for new sources of natural antioxidants that are safe, cheap and without the adverse effects of synthetic ones (Małecka, 2002). These new natural extracts should have the ability to act either directly taken as a diet complement or as an additive in food systems, fulfilling additional technological functions such as cationic dye removal (Sanchez-Martin et al., 2011) or increasing the oxidative stability of oils and oil-in-water emulsions (Kiokias, Dimakou, & Oreopoulou, 2009).

For all these reasons, tara pods have a high potential for medical, industrial and food applications. Their content in gallic acid and tannins, among other compounds, makes them a suitable raw material for preparing a wide variety of extracts. Thus, this work has two main aims: (1) to search for the best extraction method from tara pods to take advantage of their antioxidant and antiradical properties, and (2) to assess the usefulness of these extracts as oil-in-water emulsions antioxidant.

4.2 Materials and methods

4.2.1 Raw material and Reagents

Fruits pods of *Caesalpinia spinosa* (tara) were commercial product from Peru (Mabrata Tara Powder, Agrotara SAC). Refined sunflower oil was purchased in a local market.

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, phosphate buffered saline (PBS), 2.2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), 2,2,-azobis (2-methylpropionamide) dihydrochloride (AAPH), fluorescein ($C_{20}H_10Na_2O_5$) and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK). Folin-Ciocalteu reagent, absolute

ethanol, aluminum oxide, ferric chloride ($FeCl_3$), ammonium thiocyanate (NH_4SCN), anhydrous sodium carbonate and Tween 20 were analytical grade from Panreac (Barcelona, Spain). Deionized and pure water (Millipore-Q System) was used for the study.

4.2.2 Extraction

Air-dried and ground to a fine powder tara pods were weighted (3 g) and extracted with 60 mL of solvent. Water and ethanol/water mixtures at 75/25 and 50/50 (vol/vol) were selected as extraction solvents. Two different methods described below were used to check which solvent could extract the maximum amount of polyphenolic compounds:

1) The solution was stirred continuously for 24 hours at 4 °C.

2) The solution was ultrasound-processed in a bath sonicator for 1 h at room temperature.

The extracts, prepared in triplicate, were centrifuged (Sigma 6K10, Germany). Part of the supernatant was taken for subsequent use to determine the antiradical capacity. The volume of the remaining supernatant was measured and the solution was evaporated, frozen at -80 °C for 24 hours and lyophilized for 3 days. Samples were then weighed and kept protected from light in a desiccator until use to prepare an oil-water emulsion system.

4.2.3 Total phenol and flavonoid content

Total polyphenol content of extracts was determined by colorimetric spectrophotometry following the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999), slightly modified and adapted for microplates. Samples were taken from the extract solutions, diluted 1:30 (v:v) and Folin-Ciocalteu reagent (4% by volume), sodium carbonate solution 20% (30.8% by volume) and Milli-Q water were added. Samples were well mixed and left in the dark for 1 h. The absorbance was measured at 725 nm using a UV–vis spectrophotometer (Fluostrar Omega, Perkin-Elmer, Paris, France) and the results were expressed in gallic acid equivalents, GAE, using a gallic acid standard curve (10-70 µM).

Flavonoid content was determined as described by Bonvehi, Torrento & Lorente (2001) with some modifications. An appropriate dilution of the extract was mixed with the same volume of 2% $AlCl_3$ in methanol solution (5% acetic acid in methanol). The mixture was allowed to react for 10 min and the absorbance was read at 430 nm against a blank

sample without reactants. Values were determined from a calibration curve prepared with catechin (ranging from 6 to 60 mg/L) and expressed as mg of catechin equivalent per gram of dry weight.

4.2.4 Antioxidant capacity determination

4.2.4.1 ABTS assay

The first method used was the $ABTS^+$ (radical cation) discoloration assay (Re et al., 1999). The assay is based on the ability of an antioxidant compound to quench the $ABTS^+$ relative to that of a reference antioxidant such as Trolox. A stock solution of $ABTS^+$ radical cation was prepared by mixing ABTS solution with a potassium persulfate solution at 7 mM and 2.45 mM final concentration, respectively. The mixture was maintained in the dark at room temperature for 16 h before use. The working $ABTS^+$ solution was produced by dilution in 10 mM PBS (pH 7.4) incubated at 30 °C of the stock solution to achieve an absorbance value of 0.7 (±0.02) at 734 nm. An aliquot of 20 µL of diluted extract was added to $ABTS^+$ working solution (180 µL). For the blank and standard curve, 20µL of PBS or Trolox solution were used, respectively. Absorbance was measured by means of a UV–vis spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) at 734 nm immediately after addition and rapid mixing (t_0) and then every minute for 15 min. Readings at t = 0 min (t_0) and t = 5 min (t_5) of reaction were used to calculate the inhibition percentage value for each extract:

$$\%\ inhibition\ of\ the\ blank = \frac{t_0 - t_5}{t_0} 100$$

$$\%\ inhibition\ of\ the\ sample = \frac{t_0 - t_5}{t_0} 100 - \%\ inihibition\ of\ the\ blank$$

A standard reference curve was built by plotting % inhibition value against Trolox concentration (2–32µM). The radical-scavenging capacity of extracts was quantified as mmol or g of Trolox equivalent per gram of dry weight.

4.2.4.2 The oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) method was adapted from Ou, Hampsch-Woodill & Prior (2001). The assay was performed with an automated

microplate reader and 96-well plates. Diluted extract (40µL) was transferred by pipette into each well and then 120 µL of 1,34 µM fluorescein working solution in phosphate buffer (13,3 mM) and 37ºC were added to each sample. The plate was placed in a spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) and incubated at 37ºC. The initial fluorescence was recorded at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. 2,2`-Azobis(2-amidopropane)dihydrochloride (AAPH, 40 µL, 30 mM) was then added to each sample well and the fluorescence was measured immediately and every 2 min thereafter for 120 min. For the calibration curve, solutions of Trolox were prepared in the range 8-58 µM. The ORAC value for each extract was calculated using a regression equation relating Trolox concentration and the net area under the fluorescence decay curve (AUC).

$$AUC = \left(\frac{0.5 + \sum_{i=1}^{Nc} fi}{fi}\right) tc$$

$$Decrease_{fluorescence} = AUC - AUC_{Bl}$$

AUC = area under the curve of the sample in the well.

AUC_{Bl} = area under the curve of the blank

fi **=** fluorescence units (*f1,* is the value of the first reading).

Nc= number of cycles

tc: time of each cycle, in this case tc = 2 (2 minutes)

Results are expressed as mmol or g of Trolox equivalents per gram of dry weight.

4.2.4.3 FRAP assay

The FRAP method was performed as described by Benzie & Strain (1996) with some modifications. The FRAP reagent was prepared with acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl, 40 mM) and $FeCl_3$ (20 mM). The proportions were 10:1:1 (v:v:v), respectively. A suitable dilution of the extract was added to the FRAP reagent (1:30, v:v) and incubated at 37ºC. The assay was performed by means of an automated microplate reader (Fluostar Omega, Perkin-Elmer, Paris, France) with 96-well plates. The absorbance at 593 nm at time zero and after 4 min was recorded. The analysis was performed in triplicate for each triplicate extract and values were determined from a

calibration curve of Trolox (ranging from 2.5 to 33 µM). The results are expressed as mmol or g of Trolox equivalent per gram of dry weight.

4.2.5 Determination of gallic acid by high-performance liquid chromatography (HPLC)

HPLC analyses of the tara pod extracts were carried out using a Acquity UPLC System (Waters, USA) with photodioda array (PDA) detector. Tara pod extracts (10 µL) were injected on to an analytical C_{18} column (Symmetry, 5 µm, 3,9 x 150 mm, Waters) and the column temperature was set to 25°C. The mobile phase was composed of 0,5% formic acid (v/v) in acetonitrile (eluent A) and 0,5% formic acid (v/v) in water (eluent B). The gradient program was as follows: 5% A (6 min), 5-95% A (2 min), 95% A (5 min), 95-5% A (1 min), 5% A (6 min). Total run time was 20 min. The detection was made at 273 nm for gallic acid. The standard was identified by its retention time and its concentration was calculated by comparing the peak area of samples with this of the standard. Standard solutions with concentrations ranging from 10 to 100 ppm were then prepared by diluting the stock standard solution with water. The tara pod extracts obtained using different extraction conditions were filtered through a 0,45 µm filter for HPLC analysis.

4.2.6 Oil- in-water emulsion system

4.2.6.1 Antioxidant evaluation in oil-in-water emulsion

Oil-in-water emulsions were prepared with 1% of Tween 20 as emulsifier and 10% of sunflower oil triacylglycerols, previously filtered through alumina as described by Yoshida, Kajimoto & Emura (1993) in order to remove the tocopherols. The oil was added dropwise to the aqueous sample containing emulsifier cooled in an ice bath, while sonicating for 5 min in total. Freeze-dried powder of the extract of tara was redisolved in ethanol 50% (v/v) and added directly to the emulsion and homogenized, obtaining final concentrations of 12, 24 and 48 mg/L (C1, C2 and C3, respectively). For the negative control no extract was added, and the positive control was prepared with Trolox (17.8 mg/L) dissolved in ethanol. The Trolox concentration was selected since it has already been shown to exert antioxidant effect in previous study under the same conditions of storage. Emulsions were stored in triplicate in 30 mL amber bottles in the dark and allowed to oxidize at 38ºC. Peroxide value (PV) was measured periodically using aliquots of 0.005–0.1 g of each sample and determined by the ferric thiocyanate method (Frankel,

1998), after calibrating the procedure with a series of oxidized oil samples analyzed by the AOCS Official Method Cd 8-53 (1997).

The pH of the samples was measured (pH-meter GLP21, Criston Instruments, Spain) as a parameter to investigate its correlation with PV.

4.2.7 Principal Component Analysis

A Principal Component Analysis (PCA) was carried out in order to find out the correlation between the analyzed compounds and activities and the used extraction methods and solvents. This multivariate procedure was performed by means of *The Unscrambler* v. 10.1 (Camo Process AS, Oslo, Norway).

4.3 Results and discussion

4.3.1 Total polyphenols content

Total polyphenols were quantified in the extracts in order to compare the three solvents and the two extraction methods (Table 4.1). The best polyphenol extraction was achieved using ethanol 75%. Meanwhile, multiple range tests showed that no significant differences existed between *passive* (24h at the refrigerator) and *active* (1h ultrasonic) extraction with this solvent ($p>0.05$). On the contrary, the lowest polyphenol amount was extracted using water as solvent, regardless the method. In average, ethanol 75% allowed an increase of 31.4% in the performance of the extraction when compared with water. Indeed, Principal Component Analysis (in which the two first principal components explained 74% of the overall variance) revealed that phenolics content was strongly and negatively correlated with water, while the highest positive correlation was the one with ethanol 75% (Table 4.3). In addition, the lowest correlations were those concerning the extraction method.

The use of ethanol 75% allowed an average extraction of 0.467 g GAE/g of ground dry tara pods. Similarly, Veloz-García et al. (2004) reported a polyphenol extract yield of 48±3% of dry weight in cascalote (*Caesalpinia cacalaco*) pods, a plant of the same genus, using a mixture of water-methanol-acetone (8:1:1). Almost 90% of this phenolic extract was found to be gallic acid, while the remaining 10% was tannic acid. Galvez *et al.* (1997) found a yield of 25% in gallic acid (on dry weight) after a hot basic hydrolysis and acid crystallization. However, Romero *et al.* (2012) reported an extraction of only 44 mg GAE/g of ground tara pods after a process with supercritical CO_2. Consequently, the

extraction method and the used solvent play a key role in the extraction of polyphenols from *Caesalpinia sp.* pods. Likewise, Seabra *et al*. (2012) studied the extraction of phenolics from tara seeds with different pressurized CO_2-ethanol-water mixtures and stated that the best yields were also achieved with ethanol- rich solvents (extraction in different degrees of ethanol/water mixtures at 50:50 to 98:2 (vol/vol), while water and CO_2 have antagonistic effects. Whichever the considered extraction method or solvent, tara pods showed to have a remarkably higher polyphenol content than that reported for carob (*Ceratonia siliqua* L.) pods (Ayaz et al., 2007) and for *Acacia polyacantha, A. tortillis, A. nilotica* and *Dichrostachys sp.* pods (Rubanza et al., 2003) .

4.3.2 Total flavonoids content

Flavonoids represent approximately two thirds of the dietary phenolics, being also the most important group concerning their biological activities (Scalbert & Williamson, 2000). From these, the most abundant molecules in the diet are flavonols (catechins plus proanthocyanidins), anthocyanins and their oxidation products. As far as flavonoids extraction from tara pods is concerned (Table 4.1), significant differences were found depending on both the extraction method and the solvent. The highest content was obtained in the extract performed with water during 24 h in cold (3.08 mg CE/g). This method provided in all cases better results for flavonoids extraction than ultrasonication for 1 h, regardless the solvent. In the same way, pure water provided better results than ethanol, which was expected due to the fact that flavonoids are mainly water-soluble compounds (Harborne, 1998). Both facts can also be observed in the PCA correlations (Table 4.3).

Although no flavonoid concentration in tara pods has been reported to the date, it was expected to be high due to their tannins content, since chemical degradation of tannins leads to flavanols including catechin, among several others. In carob (*Ceratonia siliqua* L.) pods, a remarkably lower flavonoid content has been reported, being between 0.41 and 0.48 mg CE/g dry weight (Ayaz et al., 2007; Makris & Kefalas, 2004).

Table 4.1 Polyphenol and flavonoids content of the different tara pod extracts.

		Polyphenol content	Flavonoids content
Extraction	Solvent	g GAE/g DW	mg CE/g DW
24h refrigerator	Water	0.349 ± 0.01^{a}	3.08 ± 0.02^{f}
	EtOH 50%	0.445 ± 0.01^{c}	2.89 ± 0.03^{d}
	EtOH 75%	0.460 ± 0.02^{d}	2.93 ± 0.02^{e}
1h ultrasonic	Water	0.356 ± 0.01^{a}	2.48 ± 0.04^{c}
	EtOH 50%	0.431 ± 0.01^{b}	1.30 ± 0.03^{a}
	EtOH 75%	0.467 ± 0.01^{d}	1.79 ± 0.02^{b}

Results are expressed as mean value ± standard deviation.
Different letters within each column indicate significant differences according to multiple range tests (confidence level 95%).

Table 4.2 Antioxidant activity of the different tara pod extracts.

		Antioxidant activity					
		ABTS		ORAC		FRAP	
	Solvent	mmol TE/g DW	g TE/g DW	mmol TE/g DW	g TE/g DW	mmol TE/g DW	g TE/g DW
24h	Water	6.90 ± 0.28^{a}	1.69 ± 0.03^{a}	2.29 ± 0.28^{a}	0.57 ± 0.07^{a}	4.66 ± 0.06^{b}	1.16 ± 0.01^{b}
	EtOH 50%	7.37 ± 0.03^{b}	1.80 ± 0.04^{b}	4.19 ± 0.24^{bc}	1.05 ± 0.06^{bc}	5.99 ± 0.06^{d}	1.49 ± 0.01^{d}
	EtOH 75%	9.60 ± 0.09^{c}	2.40 ± 0.02^{c}	3.98 ± 0.38^{b}	1.00 ± 0.09^{b}	3.89 ± 0.29^{a}	0.97 ± 0.07^{a}
1h	Water	6.80 ± 0.21^{a}	1.75 ± 0.05^{a}	2.55 ± 0.42^{a}	0.64 ± 0.10^{a}	4.59 ± 0.05^{b}	1.15 ± 0.01^{b}
	EtOH 50%	6.91 ± 0.02^{a}	1.73 ± 0.01^{a}	4.22 ± 0.17^{c}	1.05 ± 0.04^{c}	5.55 ± 0.07^{c}	1.39 ± 0.02^{c}
	EtOH 75%	10.17 ± 0.20^{d}	2.54 ± 0.05^{d}	4.29 ± 0.18^{c}	1.07 ± 0.04^{c}	3.81 ± 0.12^{a}	0.95 ± 0.04^{a}

Results are expressed as mean value ± standard deviation. Different letters within each column indicate significant differences according to multiple range tests (confidence level 95%).

Table 4.3 Correlations between the analyzed compounds and activities and the extraction methods and solvents, from the Principal Component Analysis.

		Phenolics	Flavonoids	ABTS	ORAC	FRAP
	Flavonoids	-0.3253				
	ABTS	0.7392	-0.0790			
	ORAC	0.9605	-0.4853	0.5438		
	FRAP	-0.0977	-0.0709	-0.7194	0.1329	
Solvent	Water	-0.9705	0.3979	-0.5668	-0.9893	-0.1087
	EtOH 50%	0.2964	-0.3421	-0.4173	0.5237	0.9034
	EtOH 75%	0.6741	-0.0558	0.9842	0.4655	-0.7947
Method	Ultrasonic	0.0017	-0.8478	0.0020	0.1213	-0.1258
	Cold maceration	-0.0017	0.8478	-0.0020	-0.1213	0.1258

4.3.3 Antioxidant activity

The high phenolic and flavonoid content of tara pod extracts may be a good indicator of their antioxidant capacity. Antioxidant properties of phenolic compounds have been thoroughly described, although in some cases the mechanism of such activity is not fully understood yet. Some of the biological functions leading to this effect involve phenolic compounds acting as hydrogen donors, free radical acceptors, chain oxidation reaction interrupters or metal chelators (Viuda-Martoset al., 2010). On the other hand, antioxidant activity has been quantified in different extracts from pods of *Acacia pennatula* (Feregrino-Pérez et al., 2011), *Acacia auriculiformis* (Sathya & Siddhuraju, 2012), *Acacia nilotica* (Omara et al., 2012), *Ceratonia siliqua* L. (Makris & Kefalas, 2004), *Erythrina lysistemon* (Juma & Majinda, 2004), *Arachis hypogaea* L. (Lee et al., 2008), *Phaseolus vulgaris* L. (Patra et al., 2012) and *Caesalpinia cacalaco* (Veloz-García et al., 2004), among others.

Antioxidant activity of the extracts from tara pods was assessed through three different methods: $ABTS^+$, ORAC and FRAP (Table 4.2). Using several methods provide more comprehensive information about the antioxidant properties of the original product (Martínez et al., 2012). The highest functionality measured via $ABTS^+$ discoloration was found in ethanol 75% extracts, being slightly higher if the process had been carried out with ultrasound (10.17 mmol TE/g) than with cold maceration (9.60 mmol TE/g). The activity of the other extracts, regardless the solvent and the method, were remarkably lower, between 6.90 and 7.37 mmol TE/g. $ABTS^+$ quenching activity was positively correlated with total phenolics content, as well as with ethanol 75% (Table 4.3), confirming the previously stated conclusion. As far as the ORAC assay is concerned, results depended mainly on the used solvent, although the highest activity (4.29 mmol TE/g) was found again in the sample extracted with ethanol 75% with ultrasound. However, no significant differences were found between ethanol 50% and ethanol 75% in any of the two methods ($p>0.05$). Meanwhile, extracts performed with water provided the lowest ORAC values. The ORAC assay was the antioxidant assessing method best correlated with total phenolics content in the extracts (0.9605; Table 4.3). If a linear (univariate) regression is carried out between the means of phenolics content and ORAC values, the resulting model is significant with a p-value of 0.0023 and a determination coefficient (R^2) of 0.9225. Finally, opposite to what has been described for $ABTS^+$ and ORAC methods, the FRAP assay revealed the lowest ferric reducing ability of the

extracts performed with ethanol 75% (3.85 mmol TE/g on average). In addition, ethanol 50% showed better activity than the water ones, being in turn higher in the cold maceration extract (5.99 mmol TE/g). However, FRAP had the weakest correlations with phenolics and flavonoids content.

Previous reported antioxidant assays on extracts from pods of *Caesalpinia sp.* are very limited. Romero *et al*. (2012) found an activity of 0.83 μg GAE/mL of supercritical CO_2 tara polyphenol extract assessed via DPPH assay. However, as previously commented, the polyphenol content in that extract was much lower than in the present work, probably due to the used extraction method. Veloz-Garcia *et al*. (2004) described the antioxidant (*β*-carotene-linoleate method) and antiradical activity (DPPH) of the phenolics extracted from *Caesalpinia cacalaco*, finding dose-dependent, comparable values to those of pomegranate seeds.

Martinez *et al*. (2012) assessed the antioxidant activity of *Theobroma cacao* L. by means of the ABTS, DPPH and FRAP methods, obtaining in all cases lower values (in the order of μM TE/g) than those found for tara in this study. Again, also the phenolics content reported for those samples was remarkably lower (between 80.17 and 365.33 mg/100g depending on the source and the solvent). The same authors also found a good univariate correlation between total phenolics and the results of both ABTS and DPPH assays. However, also a good correlation was reported between phenolics and FRAP, which has not been found in this work. Regarding carob (*Ceratonia siliqua* L.) pods, a well-known source of antioxidant phenolics, Makris & Kefalas (2004) found that its extracts exhibited higher activity (around 1.3 mM TE) than aged red wines tannins and tannic acid, in the same scale to that of catechin and lower than that of gallic acid, quercetin and caffeic acid. However, the results found in tara pod extracts are even higher than these reported for carob pods.

4.3.4 Quantitative analysis of gallic acid

The results (Table 4.4) revealed that the gallic acid concentration ranged from 16.9 to 33.4 mg/g DW. Water was found to give the extract with the highest gallic acid content; on the contrary, when ethanol 75% was used as extraction solvent the content of gallic acid was the lowest. Extracts performed with ethanol provided the lowest gallic acid content, because the majority of extracted compounds can be derivatives of this molecule. The galloylquinic acids of tara tannin can be divided into different groups: mono, di, tri-,

and tetragalloylquinic acids that lack depsidic galloyl residues; depsides related to the 3,4,5-trigalloyl structure previously considered to be the major components of tara tannin (Clifford, Stoupi & Kuhnert 2007).

Table 4.4 Effect of different extraction on gallic acid content of tara pod extracts.

Extraction	Solvent	mg GA/g DW
24h refrigerator	Water	33.392 ± 1.8
	Ethanol (50%)	22.289 ± 0.2
	Ethanol (75%)	16.925 ± 0.6

Results are expressed as mean value ± standard deviation.

4.3.5 Oil-in-water emulsions

Besides their effects on human health through direct ingestion, one of the main applications of antioxidant extracts is their use in the food industry to avoid or delay oxidation of perishable systems. In this study, this technological ability has been assessed in oil-in-water emulsions as a model food, testing water and ethanol 75% extracts performed in cold maceration.

4.3.5.1 Peroxide value

The formation of hydroperoxides (Figure 4.1) was significantly faster in the sample without any antioxidant, reaching 40.5 meq/kg after only 4 days ($p<0.05$). This value was outreached at the 14th day in the control with 17.8 μg/mL of Trolox, while the samples with tara extracts offered a prevention from oxidation between these. As expected from the results discussed in the previous section, the extracts carried out with ethanol 75% provided better protection than water ones at the same concentration. As an example, after one week peroxide value was 122.5 meq/kg in the negative control, 100.7 meq/kg in the 12 μg/mL water-extract (W_C1), 49.3 meq/kg in the 12 μg/mL ethanol 75%-extract (Et_C1), 18.8 meq/kg in the 48 μg/mL water-extract (W_C3), 2.2 meq/kg in the 48 μg/mL ethanol 75%-extract (Et_C3) and 1.9 meq/kg in the positive control. The most remarkable issue is that, as observed in Figure 1, the behavior of the emulsion with 48 μg/mL of ethanol 75% extract is the same as the positive control with Trolox until the end of the experiment (after 18 days).

If the induction time is defined as the time in which the sample remains under 20 meq of hydroperoxides/kg, this parameter takes 12 days for the Trolox control and the Et_C3 sample, 8 days for Et_C2, 5 days for Et_C1, 4 days for W_C2 and W_C1 and 1 day for

the negative control. When the time to reach higher PV is calculated (Figure 4.2), the same order is maintained. For all values, 48 μg/mL of the extract of tara pods performed with ethanol 75% led to the same delay in oxidation as 17.8 μg/mL of Trolox. Kiokias *et al*. [20] reported peroxide values between 45.60 and 51.15 meq/kg after 2 months in 10% sunflower oil-in-water emulsions with 2 g/L of different carotenoids including *β*-carotene, lycopene, paprika, lutein and bixin. Although the time to reach these values is remarkably shorter in this study, also the concentration of antioxidant extracts is much lower (between 12 and 48 mg/L).

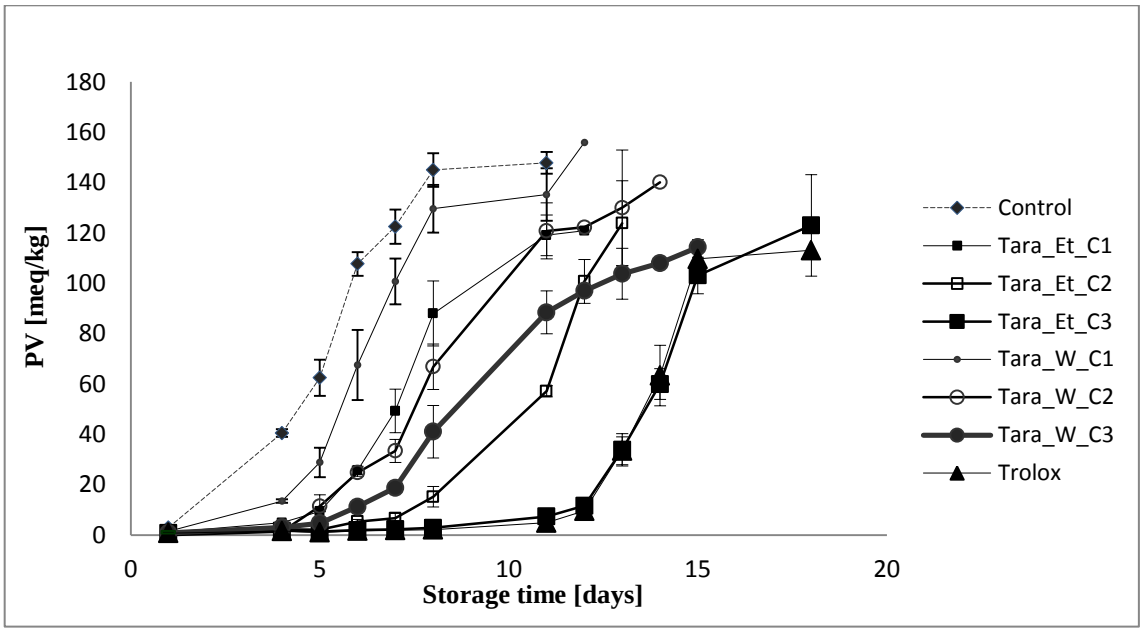

Figure 4.1 Evolution of primary oxidation (peroxide value) in model food system (O/W emulsion, 10% of oil) with different concentration of tara extracts. Et: extract performed with ethanol 75%. W: extract performed with water. C1: 12 mg/L. C2: 24 mg/L. C3: 48 mg/L.

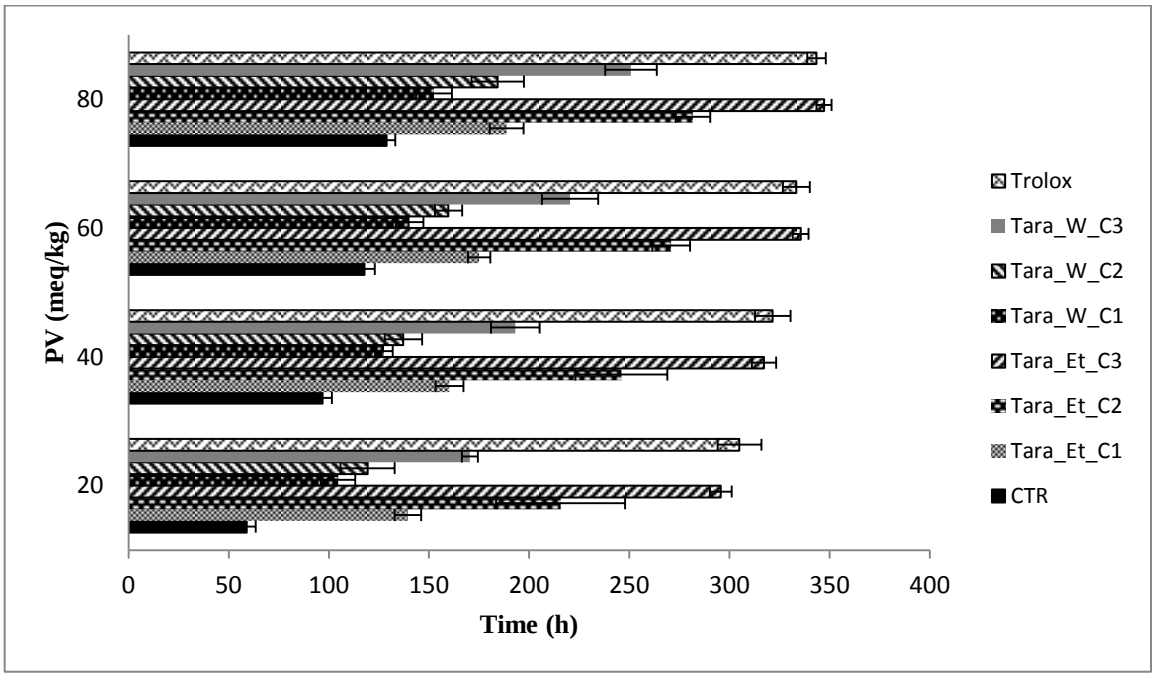

Figure 4.2 Time to reach different peroxide values (PV) in model food system (O/W emulsion 10% of oil) with different concentration of tara extracts. Et: extract performed with ethanol 75%. W: extract performed with water. C1: 12 mg/L. C2: 24 mg/L. C3: 48 mg/L.

Maisuthisakul *et al*. (2006) reported that a 10% oil-in-water emulsion with 100 mg/kg of teaw (*Cratoxylum formosum* Dyer) extract took 4.55 days at 60ºC to reach a PV of 50 meq/kg. In this work, the emulsion with 48 mg/L of the ethanol 75% extract overcame this value at the 14th day at 38ºC. Ramful *et al*. (2011) found that *Eugenia pollicina* leaf extract at a concentration of 0.02% was also effective in slowing down hydroperoxide formation in soybean oil emulsion during 13 days of storage at 40°C. Roedig-Penman & Gordon (1997) reported that tea extracts added to sunflower oil-in-water emulsion were very effective in their stabilization, the tea extract (0.03%) being similar to BHT (0.02%) and taking 40 days of storage at 30°C to reach a PV of 30 meq/kg.

4.3.5.2 pH

Hydroperoxides are the main primary products of lipid oxidation, but they are highly unstable and easily break up into secondary compounds, resulting in the appearance of aldehydes, ketones, epoxides or organic acids, which may lead to changes in the pH (Sun, Wang, Chen, & Li, 2011). In addition, since it is known that many antioxidant molecules are less effective when the pH is low (Zhou & Elias, 2013), and oxidative reactions are reaction-type, this parameter was also measured as a potential indicator of oil-in-water emulsions oxidation. From an initial average value of 6.57, all samples tended to stabilize

their pH at around 3.03 after 18 days. The behavior of pH (shown in Figure 4.3) was the opposite of PV, being the positive control and the tara Et_C3 sample the ones that kept their pH stable for longer (up to 8 days above 6.30) before decreasing. The Et_C2 (24 μg/mL) pH also remained stable until that time, but decreased rapidly after. In this case, if induction time were defined as the time in which the sample pH remains above 6, it was found to be 12 days for the Trolox control and the Et_C3 sample, 8 days for Et_C2, 7 days for Et_C1, 5 days for W_C2 and W_C1 and 2 days for the negative control. Therefore, for the most stable samples (Trolox control, Et_C3 and Et_C2) this induction time is the same as for peroxide value, while for the least stable ones pH begins changing slightly later.

Observing this inverse relationship between the behavior of PV and that detected for pH, a regression was carried out on the means of the values for the 8 different tested emulsions (2 controls and 6 tara pod extracts) during 18 days (Figure 4.4). A negative exponential relationship was found between both parameters, with an equation of $pH = 6.80 \cdot e^{-0.006 \cdot PV}$ and R^2=0.9648.

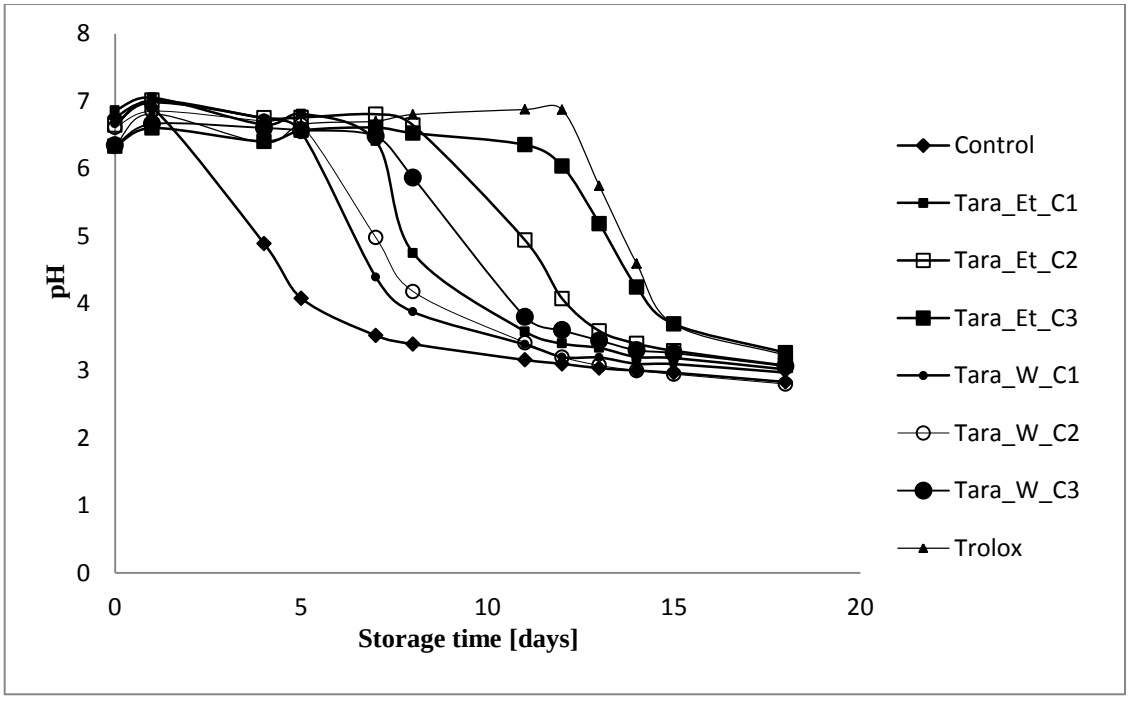

Figure 4.3 Evolution of pH in model food system (O/W emulsion, 10% of oil) with different concentration of tara extracts. Et: extract performed with ethanol 75%. W: extract performed with water. C1: 12 mg/L. C2: 24 mg/L. C3: 48 mg/L.

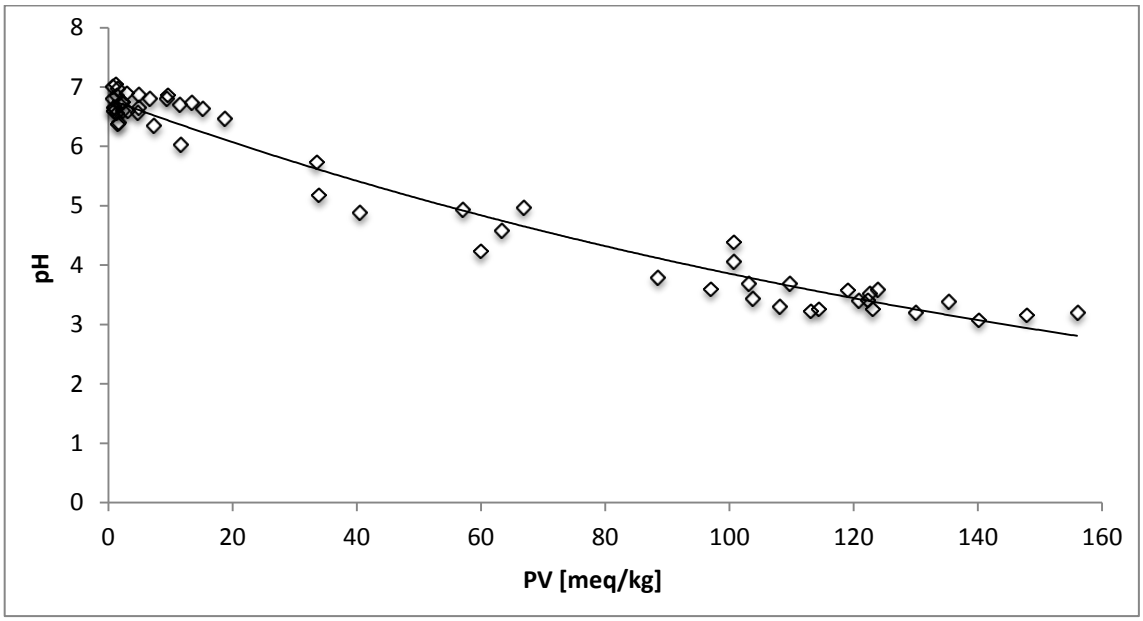

Figure 4.4 Peroxide value-pH regression for the oil-in-water emulsions oxidation (means of 3 replicates for 8 different emulsions including 6 with tara extracts, positive control with Trolox and negative control) during 18 days. Continuous line belongs to the regression equation $pH = 6.80 \cdot e^{-0.006 \cdot PV}$, with R^2=0.9648.

The literature on lipid oxidation published in the past years has been extensive; however, some of the references that compare the effect of various pH on lipid oxidation are conflicting (Sun et al., 2011). Mancuso *et al*. (1999a, 1999b) reported that the initial oxidation of emulsion was pH-dependent, with a varying effect for different emulsifiers. They observed a higher oxidation rate at pH 7 than at pH 3, especially in o/w emulsions stabilized with 1% Tween 20. The faster increase of primary oxidation products at higher pH could be caused by increased formation or decreased degradation of these products, or by a combination of both factors. This low pH can increase the solubility of iron and more iron can be partitioned into the continuous phase, whereas at high pH insoluble iron precipitates onto the emulsion droplet surface, and may increase lipid oxidation rates because of the close proximity of the iron and lipid substrate. Donnelly *et al*. (2006) reported that oxidation of a whey protein isolate (WPI)-stabilized emulsion decreased with decreasing pH (3-7) but in a Tween 20 stabilized emulsion, oxidation increased with decreasing pH. Also Sorensen et al. (2008) reported that lipid oxidation increased when pH was decreased from 6 to 3 in a 10% oil-in-water emulsion.

4.4 Conclusions

Tara (*Caesalpinia spinosa*) pods are an outstanding source of phenolic compounds and flavonoids, which can be extracted in different degrees using water or ethanol/water mixtures. The extracted amount of phenolics and flavonoids, as well as their antioxidant activity, depend on both the solvent and the extraction method. In this way, the use of a solution of ethanol 75% in a 1-h ultrasonic process allows achieving the greatest quantity of phenolics (0.4676 g GAE/g DW) and provides the best antioxidant activity measured by the $ABTS^{+}$ and ORAC methods (10.17 and 4.29 mmol TE/g DW, respectively). The highest amount of flavonoids is extracted with a 24-h maceration in cold water, and 24-h cold maceration in ethanol 50% leads to the extract with the highest antioxidant activity assessed through the FRAP method. Tara extracts obtained with ethanol 75% can be applied as antioxidants in oil-in-water emulsions. The addition of 48 μg/mL of this extract to the emulsion with 10% oil delays oxidation to the same extent as 17.8 μg/mL of Trolox. The oxidative process of the emulsion can be assessed either by measuring the peroxide value or the pH, since both parameters are strongly related by an exponential-type equation.

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5 Effect of tara (*C. spinosa*) pods on the quality and shelf-life stability of model meat products

5.1 Introduction

Lipid oxidation is the main cause of deterioration and reduced shelf life of cooked meat (Lara et al., 2011; Sánchez-Escalante et al., 2011). This process may produce changes in meat quality parameters such as colour, flavour, odour, texture, and even nutritional value. The heating process leads to increased oxidation of lipids in meat, which cause a warmed-over flavour in chilled cooked meat products (Brewer, 2009; Hayes et al., 2011). The rate and extent of oxidative deterioration can be reduced through various procedures like curing, vacuum packaging, modified atmosphere packaging and, most importantly, adding synthetic or natural antioxidants (Banerjee et al., 2012). Although synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate and tertiary butylhydroquinone (TBHQ) have been used extensively, recent studies have implicated them to have various negative health effects on animals and human cells (Pop et al., 2013). Concerns regarding the safety and toxicity of synthetic antioxidants have promoted research into natural antioxidants derived from plant sources (such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs) (Bastida et al., 2009; Brewer, 2011; Cofrades et al., 2011). In recent times, the functional antioxidant properties of numerous plant extracts containing phenolic compounds have been investigated in cooked meat products (Choe et al., 2011; Qin et al., 2013; Tapp et al., 2012).

Tara pod extracts rich in gallic acid and tannins were successfully applied to increase the oxidative stability of oils (Chambi et al., 2013; Romero et al., 2012) and oil-in-water emulsions (Skowyra et al., 2014). Although not yet reported in the literature tara pods could therefore be incorporated in cooked meat products as a source of natural antioxidants to prolong quality and stability. For all these reasons, the aim of the present study was to evaluate the effect of tara pods on lipid oxidation, colour stability and textural properties of cooked model meat systems stored at 4ºC for 21 days.

5.2 Materials and methods

5.2.1 Plant and meat material

Fruit pods of *Caesalpinia spinosa* (tara) were a commercial product from Peru (Mabrata Tara Powder, Agrotara SAC). The dried powder of tara pods was stored in darkness, at room temperature. Pork ham and pork back fat were obtained from a meat plant "Edward and Grzegorz Dworeccy" placed in Golejewo (Poland). The meat was trimmed of visible fat and connective tissue. Lots of approximately 500 g were packed, frozen and stored at -20ºC until used.

5.2.2 Meat model system formulation and processing

Meat and back fat packages were thawed (approximately 18 h at 3±2ºC). After this the materials were passed through a grinder with a 0.6 cm plate (Zelmer 887.84, Poland). Five different meat model system (meat batters) formulations were made up with the same amounts of pork ham (55%), pork back fat (24%), curing salt (Solino S.A., Inowroclaw, Poland) (1.6%) and iced water (19.4%). A control formulation (control) was prepared with no added antioxidant. Three other formulations were prepared with three different levels of dried tara pod powder: 0.02% (T1), 0.04% (T2) and 0.08% (T3) (0.2, 0.4 and 0.8 g/kg of total weight of the meat product). The last meat batter (BHA) was prepared with a synthetic antioxidant, butylated hydroxyanisole (Sigma-Aldrich, St. Louis, MO, USA) (0.02%). A total of 15 batches (5 treatments × 3 replications) of pork batters were manufactured. All ingredients were placed in a cutter (Büchi Mixer B-400, Labortechnik GmbH, Essen, Germany) and mixed for approximately 5 seconds (9000 rpm). It was necessary to repeat homogenization 5 times for every batch. Finally, samples were manually stuffed into the polypropylene tubes (diameter of 30 mm). The batters were then held for 2 h at 4ºC to allow the ingredients to equilibrate. The batters were cooked by immersion in water bath until the temperature of products reached 72ºC (measured with a thermometer inserted into the centre of the batter). The cooked batters were cooled down in the ice, then taken out of the plastic tubes, dried, packaged in polyethylene bags and stored at 4ºC for 1, 7, 14 and 21 days.

5.2.3 Proximate composition and pH

Moisture and fat content of final products were determined in triplicate (AOAC 2000). Protein content was measured using Nitrogen Analyzer Kjeltec TM 2300 (*FOSS*,

Hilleroed, Denmark) in duplicate. Acidity of model meat products was measured directly using a pH meter Orion 3-Star pH Benchtop Meter (Thermo Fisher Scientific, Waltham, MA, USA).

5.2.4 Cooking loss

Cooking losses were determined immediately after the production and were expressed as weight differences of samples before and after cooking process. Before evaluation the final products were dried using a paper towel.

5.2.5 Colour measurement

The surface colour of model meat products was evaluated using a reflectance colorimeter Minolta CR-400 (Konica Minolta, Tokyo, Japan) and it was expressed against the scale of L* (lightness) a* (redness) b* (yellowness) in the CIE LAB colour space system. Before each measuring session (light source of D65 and 10º standard observer) the instrument was calibrated (white reference: Y = 93.8; x = 0.315; y = 0.332). Colour measurement was conducted six times for each variant of meat products directly after production. The evaluation was repeated after 7, 14 and 21 days of cool storage in the darkness. Also, the slices of pork products were displayed under white fluorescent light (250 lx) at 4ºC for 48 h, simulating retail display conditions, and the colour measurement was carried out after 1 h, 3 h, 6 h, 24 h and 48 h.

5.2.6 Lipid oxidation

The effect of tara pods on lipid oxidation of the chilled batters (initially and after 7, 14 and 21 days of storage) was evaluated using a spectrophotometric 2-thiobarbituric acid (TBA) extraction method described by Grau et al. (2000) with slight modifications. Briefly, the TBARs reagent was prepared by mixing 15% (w/v) trichloroacetic acid and 0.375% (w/v) 2-thiobarbituric acid in 0.25 N hydrochloric acid. The procedure was as follows: 1 g of each sample was weighed in a centrifuge tube, and 1 ml of 0.3% aqueous EDTA (Sigma-Aldrich, St. Louis, MO, USA) was added immediately to stop progressing fat oxidation. Then, just before homogenization, 5 ml of TBARs reagent was added to the tube, and the content was homogenized for 1 min at 14 500 rpm using a Miccra D-1 Homogenizer (ART Prozess & Labortechnik GmbH & Co., Mullheim, Germany), covered, placed in a boiling water bath for exactly 10 min and then cooled for 30 min at room temperature. The mixture was centrifuged (Sigma 3K30, Sigma Laborzentifugen

GmbH, Osterode am Harz, Germany) at room temperature at 4000 rpm for 10 min. The absorbance of supernatant was measured at 532 nm (Spectrophotomter UV-1800, BRAIC, China). The TBARs values were expressed as mg of malondialdehyde (MDA) per kg of sample calculated using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, St. Louis, MO, USA) as the standard. TBARs determinations for each sample were performed in triplicate.

5.2.7 DPPH free radical scavenging capacity

The antioxidant capacity of pork batters was evaluated in extracts obtained following the procedure of Amarowicz et al. (2004) with slight modifications. Briefly, 2 g of sample was homogenized for 1 min (Miccra D-1, ART Prozess & Labortechnik GmbH & Co., Mullheim, Germany) with 5 ml of ethanol. After mixing, the sample was centrifuged (Sigma 3K30, Sigma Laborzentifugen GmbH, Osterode am Harz, Germany) at 4500 rpm for 10 min and the supernatant was separated. Finally, 0.5 ml of the diluted meat extract was mixed with 1.5 ml of ethanol and 0.5 ml of 0.12 mg/ml DPPH (Sigma-Aldrich, St. Louis, MO, USA) ethanol solution. The reaction mixture was shaken thoroughly and then left in the dark at room temperature for 10 min. Absorbance was measured at 517 nm (Spectrophotometer UV-1800, BRAIC, China). The ability to scavenge the DPPH free radical was calculated with the following equation:

$$Inhibition\ \% = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100$$

where $Abs_{control}$ is the absorbance of the DPPH free radical ethanol and Abs_{sample} is the absorbance of the DPPH free radical solution containing the sample extract. Trolox (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard, and results were expressed in Trolox equivalents (mmol TE/g sample). All determinations were performed in triplicate.

5.2.8 Texture profile analysis

The texture profile analysis (TPA) of model meat products was conducted using Zwick/Roell Z010 testing machine (Zwick Testing Machines Ltd**.,** Leominster, Herefordshire, UK) and TPA 50 test (50% deformation, head speed 60 mm/min,

relaxation time - 30 s). The samples (slices of 15×25 mm) were compressed twice to 50% of their original height. The textural parameters of hardness, cohesiveness, springiness, gumminess and chewiness were measured (Bourne, 1978). The evaluation was conducted at room temperature (22±1°C), directly after production process and after 2 weeks of storage.

5.2.9 Statistical analysis

Mean values for different parameters were calculated and compared by analysis of variance (two-way ANOVA) using the Statistica software version 10 (StatSoft, Tulsa, Oklahoma, USA). Moreover, statistical differences between mean values were identified by Duncan test at the 95% of confidence level ($p<0.05$).

5.3 Results and discussion

5.3.1 Proximate composition, pH and cooking loss

Table 5.1 shows chemical composition, pH and technological parameters of cooked pork batters. The differences in moisture, fat and protein content among samples of cooked pork sausage were not significant. pH value of the sample T3 was significantly lower than that of other samples and the control, which could be attributed to the fact that the concentration of tara powder rich in gallic acid in these samples was the highest.

Table 5.1 Proximate composition, pH and cooking loss of cooked pork batters.

Treatment[1]	Moisture (%)	Fat (%)	Protein (%)	pH	Cooking loss (%)
Control	56.31 ± 2.10	21.24 ± 0.89	13.35 ± 0.26	6.45 ± 0.02^{ab}	6.09 ± 0.65^{ab}
BHA	56.40 ± 1.18	21.77 ± 0.43	13.32 ± 0.15	6.46 ± 0.02^{a}	5.15 ± 0.41^{a}
T1	58.00 ± 0.69	20.37 ± 0.14	13.48 ± 0.43	6.45 ± 0.01^{ab}	5.67 ± 0.26^{ab}
T2	58.17 ± 0.92	20.84 ± 0.27	13.85 ± 0.84	6.45 ± 0.01^{ab}	5.95 ± 0.03^{ab}
T3	57.64 ± 2.32	21.54 ± 0.13	13.58 ± 0.02	6.39 ± 0.06^{b}	6.33 ± 0.22^{b}

All values are mean ± standard deviation of three replicates.
[a-b] Means in the same column (variants) with different lowercase letters are significantly different ($p<0.05$).
[1]Control: meat batter without antioxidants; BHA: meat batter with 0.02% BHA ; T1: meat batter with 0.02% of tara powder; T2: meat batter with 0.04% of tara powder; T3: meat batter with 0.08% of tara powder

Cooking loss measures the ability of the system to bind water and fat after protein denaturation and aggregation (Hayes et al., 2011). Cooking weight losses are one of the main parameters which affect meat quality and can be mainly ascribed to water exudates

(Chiavaro, Rinaldi, Vittadini, & Barbanti, 2009). Cooking loss ranged from 5.15±0.41 to 6.33±0.22, and sample T3 showed the highest cooking losses in comparison to the rest of the batters ($p<0.05$). However, there were no significant differences in cooking losses of samples BHA, T1, T2, and the control, nor between T1, T2, T3 and the control. The cooking loss of control sample was the same as in the casa of samples with antioxidants (synthetic and natural one).

5.3.2 Instrumental colour measurement

Instrumental colour parameters measured at the surface of the cooked pork batters during 21 days of refrigerated storage are shown in Table 5.2. Lightness (L*) values of samples BHA, T1 and T2 decreased ($p<0.05$) during 21 days of storage.

Table 5.2 The colour values of cooked pork batters during refrigerated storage for 21 days.

	Treatment[1]	Storage time (days)			
		1	7	14	21
*L**	Control	72.60 ± 1.84^{aB}	71.19 ± 1.01^{bA}	71.60 ± 1.16^{aAB}	71.94 ± 0.22^{aAB}
	BHA	72.72 ± 1.02^{aB}	72.50 ± 0.51^{cB}	71.47 ± 0.20^{aA}	71.46 ± 1.23^{aA}
	T1	69.90 ± 0.93^{bB}	70.37 ± 0.55^{aB}	68.92 ± 0.25^{bA}	68.50 ± 1.34^{bA}
	T2	71.38 ± 1.40^{cB}	70.11 ± 0.05^{aA}	70.18 ± 0.81^{cA}	70.10 ± 0.54^{cA}
	T3	72.85 ± 0.52^{aA}	70.67 ± 0.45^{abB}	72.15 ± 2.26^{aA}	73.11 ± 0.70^{dA}
*a**	Control	6.11 ± 0.19^{aB}	7.73 ± 0.91^{bA}	7.31 ± 0.93^{cA}	7.33 ± 1.19^{aA}
	BHA	6.42 ± 0.97^{aB}	7.22 ± 0.69^{cA}	7.88 ± 1.04^{acA}	8.01 ± 1.40^{aA}
	T1	6.98 ± 0.23^{bB}	7.97 ± 0.34^{abC}	8.54 ± 0.78^{bA}	8.96 ± 0.80^{bA}
	T2	7.14 ± 0.34^{bC}	8.17 ± 0.48^{aA}	8.47 ± 0.73^{abAB}	8.82 ± 0.58^{bB}
	T3	7.59 ± 0.54^{cA}	8.39 ± 0.12^{aB}	8.23 ± 0.31^{abB}	7.14 ± 1.44^{aA}
*b**	Control	8.73 ± 0.54^{bB}	7.94 ± 0.52^{bA}	8.03 ± 0.54^{aA}	8.19 ± 0.43^{bA}
	BHA	8.52 ± 1.38^{bB}	7.66 ± 0.43^{bA}	7.99 ± 0.07^{aA}	8.18 ± 0.06^{abAB}
	T1	7.85 ± 0.57^{aB}	7.05 ± 0.70^{aA}	7.09 ± 0.08^{bA}	7.36 ± 0.20^{cA}
	T2	7.58 ± 0.67^{aB}	6.71 ± 0.43^{aA}	7.01 ± 0.33^{bA}	7.76 ± 0.24^{aB}
	T3	7.29 ± 0.53^{aAB}	6.81 ± 0.40^{aA}	7.71 ± 0.21^{aB}	8.99 ± 0.68^{dC}

*L** lightness; *a** redness; *b** yellowness
All values are mean ± standard deviation of three replicates.
[a-d] Means in the same column (variants) with different lowercase letters are significantly different ($p<0.05$).
[A-C] Means in the same line (storage time) with different capital letters are significantly different ($p<0.05$).
[1]Control: meat batter without antioxidants; BHA: meat batter with 0.02% BHA ; T1: meat batter with 0.02% of tara powder; T2: meat batter with 0.04% of tara powder; T3: meat batter with 0.08% of tara powder

The decrease of L* value for pork meat products was also observed by Wojciak et al. (2014) in organic pork sausages. On the other hand, L*-values of T3 and control sample was stable during this period of storage. The most important colour parameter for meat

products is the redness (a*) value. Measured a*-values ranged from 6.11 to 7.59 immediately after packaging to 7.14 to 8.96 after 21 days of storage. The samples T1 and T2 had significantly ($p<0.05$) higher redness values compared to the BHA and control samples at day 21 of refrigerated storage. Yellowness (b*) values of samples T1 and T2 were significantly ($p<0.05$) lower than BHA and control samples during all storage time. On the other hand, redness values of the cooked batters stored under illumination at 4ºC for 48 h, (conditions similar to those in supermarkets and evaluated after 1 h, 3 h, 6 h, 24 h and 48 h) ranged from 6.15 to 7.59 immediately after packaging to 3.10 to 5.19 after 48 h of storage (Table 5.3). The a*-value (5.19) of the sample T1 was significantly ($p<0.05$) higher than other samples after 48 h of storage under illumination. In addition, this sample had significantly ($p<0.05$) lower lightness and yellowness values than control samples (without antioxidant and with BHA) after 48 h of storage under illumination.

Table 5.3 The colour values of cooked pork batter under illumination at 4ºC for 48 h.

	Treatment[1]	Storage time (h)				
		1	3	6	24	48
*L**	Control	73.28 ± 1.40^{aAB}	73.01 ± 1.70^{aAB}	73.61 ± 1.34^{aB}	73.09 ± 2.12^{aAB}	72.25 ± 0.81^{aA}
	BHA	73.72 ± 0.03^{aC}	72.66 ± 0.50^{aA}	73.37 ± 1.10^{aBC}	73.03 ± 1.24^{aAB}	72.62 ± 0.12^{aA}
	T1	70.77 ± 0.09^{bAB}	70.43 ± 0.68^{bAB}	71.39 ± 0.42^{bA}	70.61 ± 1.91^{bAB}	69.88 ± 0.52^{bB}
	T2	71.84 ± 1.70^{bA}	71.71 ± 1.72^{cA}	72.27 ± 0.66^{bcA}	71.73 ± 1.43^{cAb}	70.63 ± 1.00^{bA}
	T3	73.19 ± 0.48^{aA}	73.17 ± 0.63^{aA}	72.98 ± 0.74^{acA}	72.56 ± 1.71^{acA}	71.33 ± 1.03^{cB}
*a**	Control	5.30 ± 0.37^{aD}	4.72 ± 0.19^{cA}	4.72 ± 0.01^{cA}	4.05 ± 0.18^{aC}	3.10 ± 0.55^{aB}
	BHA	5.17 ± 0.41^{aD}	4.49 ± 0.11^{bA}	4.46 ± 0.23^{bA}	4.01 ± 0.25^{aC}	3.40 ± 0.07^{aB}
	T1	6.29 ± 0.49^{bcD}	5.73 ± 0.34^{aB}	5.61 ± 0.06^{aAB}	5.46 ± 0.10^{cA}	5.19 ± 0.29^{cC}
	T2	6.17 ± 0.33^{bD}	5.65 ± 0.27^{aA}	5.53 ± 0.31^{aA}	5.05 ± 0.37^{bC}	4.04 ± 0.85^{bB}
	T3	6.44 ± 0.28^{cD}	5.76 ± 0.03^{aA}	5.55 ± 0.16^{aA}	4.97 ± 0.39^{bC}	4.31 ± 0.68^{bB}
*b**	Control	9.44 ± 0.48^{bA}	9.50 ± 0.84^{cA}	9.45 ± 0.88^{bA}	9.63 ± 0.57^{bA}	9.85 ± 0.88^{aA}
	BHA	9.38 ± 1.17^{bAB}	9.38 ± 1.38^{cAB}	9.21 ± 1.45^{bAB}	9.70 ± 1.15^{bv}	9.74 ± 1.10^{aA}
	T1	8.66 ± 0.55^{aA}	8.74 ± 0.87^{bA}	8.56 ± 1.04^{aA}	9.02 ± 0.76^{aA}	8.91 ± 0.64^{bcA}
	T2	8.47 ± 0.52^{aA}	8.66 ± 0.68^{abA}	8.45 ± 0.76^{aA}	8.62 ± 0.62^{aA}	8.59 ± 0.25^{bA}
	T3	8.38 ± 0.36^{aA}	8.45 ± 0.49^{aA}	8.24 ± 0.60^{aA}	8.53 ± 0.16^{aA}	9.25 ± 0.76^{acC}

*L** lightness; *a** redness; *b** yellowness
All values are mean ± standard deviation of three replicates.
[a-d] Means in the same column (variants) with different lowercase letters are significantly different ($p<0.05$).
[A-D] Means in the same line (storage time) with different capital letters are significantly different ($p<0.05$).
[1]Control: meat batter without antioxidants; BHA: meat batter with 0.02% BHA ; T1: meat batter with 0.02% of tara powder; T2: meat batter with 0.04% of tara powder; T3: meat batter with 0.08% of tara powder

The colour of food at the retail level exerts a strong influence on the consumer's decision to purchase. Keeping colour attractiveness is of primary importance since colour is the first attribute consumers use to evaluate meat quality and therefore it plays a major role in

influencing purchase decisions (Sánchez-Escalante et al., 2011). Meat colour is determined by its heme pigments concentration, their oxidation-reduction state and light-scattering properties (S. Brewer, 2004). In the present study, the addition of tara powder (T1 and T2) resulted in redder coloration in meat product in comparison to control (without antioxidants and with BHA) during the entire storage period (21 days and also 48 h under illumination) ($p<0.05$). In addition, samples treated with tara powder reduced L*-values and b*-values in comparison to control (without antioxidants and with BHA) ($p<0.05$) during storage (21 days and also 48 h under illumination). Thus, the addition of tara changed the colour attributes of cooked pork batters by increasing redness and decreasing lightness and yellowness.

The stabilising effects of natural antioxidants on colour has been observed and verified in several studies with other natural antioxidants such as rosemary extracts (Lara et al., 2011) or ellagic acid in cooked pork (Hayes, Stepanyan, O'Grady, Allen, & Kerry, 2010).

5.3.3 Lipid oxidative stability

The effect of tara powder on TBARs values in cooked pork batters during storage at 4ºC for 21 days is shown in fig. 5.1. In general, storage time has a significant influence on lipid oxidation in the cooked pork batters.

TBARs values in all samples containing tara additive and in BHA sample were considerably lower ($p<0.05$) than in the control (without antioxidants). Tara powder showed high protection against lipid oxidation in cooked pork batters and no differences were observed between T1, T2 and T3. The lowest concentration of tara (0.02%) in cooked pork batters showed strong lipid stabilisation during storage, similar to BHA at the same concentration (0.02%). Initial (day 1) TBARs values for all treatments were significantly lower than those in control cooked pork batter ($p<0.05$) suggesting that the antioxidants retarded lipid oxidation during and immediately after cooking. In all treated samples (T1, T2, T3) and in BHA sample, TBARs values were at the same level at day 21 (0.190-0.227 mg MDA/kg product) as in the control sample at day 1 (0.214 mg MDA/kg product).

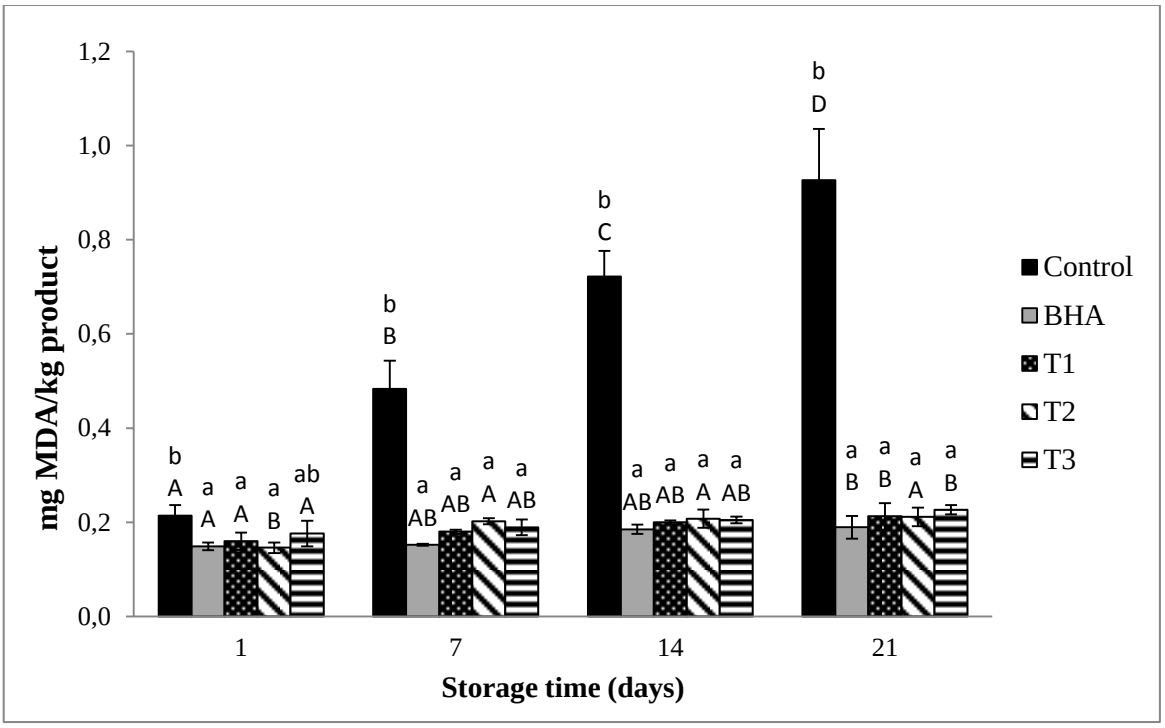

Figure 5.1 The TBARs values of cooked pork batters during refrigerated storage for 21 days.
[a-b] Values with different letters in the same batch are significantly different ($p<0.05$).
[A-C] Values with different letters at the same day of storage are significantly different ($p<0.05$).
Control: meat batter without antioxidants; BHA: meat batter with 0.02% BHA ; T1: meat batter with 0.02% of tara powder; T2: meat batter with 0.04% of tara powder; T3: meat batter with 0.08% of tara powder.

These oxidation reduction effects of tara pods may be due to the presence of phenolic compounds such as phenolic acids. Tara pod extracts were reported to contain free gallic acid and gallotannins (1.7 and 50.4 g GAE/100 g, respectively). These two compounds represented ~95% of the total phenolics present in tara pods. The remaining 5% of tara phenolics might be composed of ellagitannins and other phenolic compounds (Chambi et al., 2013). Romero et al. (2012) found that tara pod extract obtained by supercritical fluid extraction with CO_2 had high antioxidant activity and was efficient in inhibiting rancidity deterioration of sunflower oil by improving its stability. Skowyra et al. (2013) also reported a high level of antioxidant protection in oil-in-water emulsion with addition of tara extracts obtained with 75% ethanol. The addition of 48 µg/mL of this extract to the emulsion with 10% oil delayed oxidation to the same extent as 17.8 µg/mL of Trolox. Bastida et al. (2009) reported other extracts rich in condensed tannins from carob fruit which were successfully applied to reduce fat deterioration in cooked meat during chilled and frozen storage. Retardation of lipid oxidation by different plants was demonstrated also using lotus leaf powder (0.1 and 0.5%) in cooked ground pork (Choe et al., 2011). Similar results were shown with another natural antioxidants such as rosemary extracts

(0.03%) (Lara et al., 2011) or Radix puerariae extracts (1%) in precooked pork sausage (Jung, Yun, Go, Kim, & Seo, 2012).

5.3.4 DPPH free radical scavenging activity

Besides the measurement of the intensity of the oxidation process through the analysis of secondary oxidation products, the analysis of the potential antioxidant activity was also carried out. The antioxidant activity measured by the DPPH assay in cooked pork batters is presented in fig. 5.2. The highest DPPH free radical scavenging activity was found ($p<0.05$) in the T3 sample followed by T2, T1 and BHA at all analysed storage times. In all samples longer storage times caused reduction of DPPH radical scavenging activity because of the protein denaturation and the degradation of antioxidant compounds (Berasategi et al., 2011). However, measured activity was higher in T2 and T3 at day 21 (0.57 ± 0.16 and 1.40 ± 0.01 mmol Trolox/g of product, respectively) than in the control batters (without antioxidants and with BHA) at day 1 (0.39 ± 0.02 and 0.46 ± 0.01 mmol Trolox/g of product, respectively).

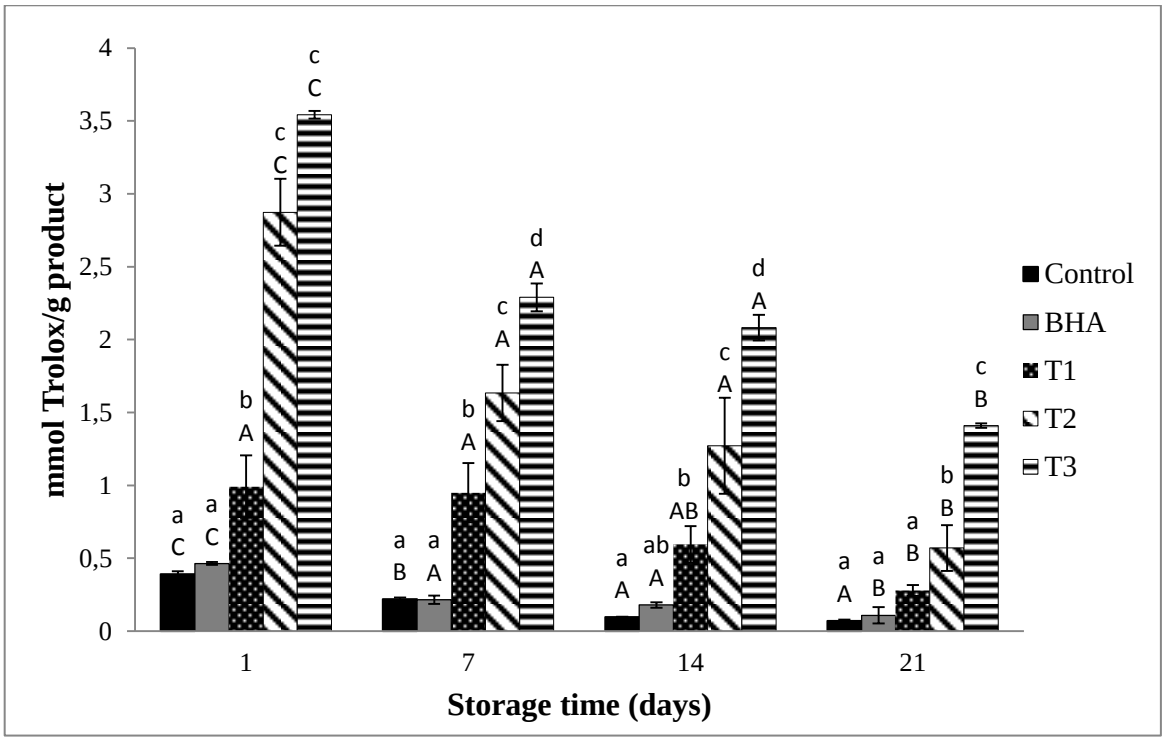

Figure 5.2 DPPH radical scavenging activity (mmol Trolox/g product) of cooked pork batters during refrigerated storage for 21 days.

[a-d] Values with different letters in the same batch are significantly different ($p<0.05$).

[A-C] Values with different letters at the same day of storage are significantly different ($p<0.05$).

Control: meat batter without antioxidants; BHA: meat batter with 0.02% BHA ; T1: meat batter with 0.02% of tara powder; T2: meat batter with 0.04% of tara powder; T3: meat batter with 0.08% of tara powder

When figures 1 and 2 are compared, the former shows that BHA sample had the same effect as the three concentration of tara pod powder on lipid oxidation. However, fig. 5.2 shows that sample with BHA had similar DPPH free radical activity as control sample, and the meat batters with three concentration of tara presented different activity. Tara pods contain high tannin concentration (~40-60% (w/w)) and many compounds with documented biological activities, such as gallotannic, tannic and gallic acids (Castaneda et al., 2012; Chambi et al., 2013; Marienfeld et al., 2003), which might have contributed to higher DPPH free radical scavenging activity in the developed pork products than synthetic antioxidant (BHA).

5.3.5 Instrumental texture

The addition of tara powder to cooked pork batters reduced ($p<0.05$) texture parameters such as: hardness, chewiness, gumminess and cohesion (Table 5.4) in comparison to control (without antioxidants and with BHA) samples at days 1 and 14.

Lipid and protein oxidation are closely associated deteriorative processes occurring in meat products during storage. It has been reported that protein oxidation can negatively affect the sensory quality of meat products in terms of texture, tenderness and colour (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). Some authors have related an increase in hardness during storage of fresh pork to higher intensity of protein oxidation reactions, leading to the formation of crosslinking bonds and polymerization in proteins (Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007). Results suggest that tara may have reduced the hardness of cooked pork product through its protective role against oxidation. Furthermore, these results indicate that the addition of tara powder was efficient in preparing precooked pork products with softer textural properties.

Cohesiveness value measures degree of difficulty in breaking down the internal structure of the sausage while springiness value represents the extent of recovery of sausage height and sometimes is referred to as “elasticity” (Jung et al., 2012). The springiness values of treated samples were considerably lower ($p<0.05$) than that of the negative control on day 1, but after 14 days of chilled storage, the values in batters containing antioxidants (BHA and tara) showed an increasing trend.

Table 5.4 Instrumental texture of cooked pork batter at day 1 and 14 of refrigerated storage.

Treatment[1]	Storage days	Hardness [N]	Springiness [mm]	Cohesiveness [-]	Chewiness [Nm]	Gumminess [N]
Control	1	49.14 ± 3.98^{ab}	2.81 ± 0.22^{d}	0.59 ± 0.03^{d}	41.28 ± 3.37^{e}	27.37 ± 0.76^{cd}
BHA		48.77 ± 2.88^{ab}	1.58 ± 0.14^{c}	0.54 ± 0.06^{cd}	33.72 ± 3.04^{bcd}	26.43 ± 3.51^{cd}
T1		38.85 ± 4.09^{a}	2.20 ± 0.25^{a}	0.42 ± 0.06^{ab}	26.79 ± 0.58^{ab}	15.25 ± 2.83^{a}
T2		41.72 ± 2.44^{a}	1.88 ± 0.10^{abc}	0.41 ± 0.07^{a}	29.17 ± 3.01^{abc}	17.33 ± 1.91^{ab}
T3		37.73 ± 4.38^{a}	1.97 ± 0.51^{ab}	0.42 ± 0.07^{ab}	27.09 ± 3.10^{ab}	17.59 ± 1.26^{ab}
Control	14	56.18 ± 5.10^{e}	1.94 ± 1.94^{abc}	0.53 ± 0.05^{cd}	51.74 ± 2.83^{f}	30.59 ± 1.85^{d}
BHA		52.81 ± 5.37^{ce}	1.76 ± 1.76^{bc}	0.49 ± 0.06^{bc}	38.23 ± 4.67^{de}	24.24 ± 2.84^{c}
T1		45.34 ± 5.33^{bd}	2.05 ± 0.12^{ab}	0.43 ± 0.04^{ab}	35.87 ± 4.04^{cde}	19.58 ± 2.88^{b}
T2		39.40 ± 1.52^{a}	2.14 ± 0.23^{ab}	0.43 ± 0.05^{ab}	27.59 ± 3.08^{ab}	17.06 ± 2.27^{ab}
T3		39.79 ± 1.52^{a}	2.23 ± 0.02^{a}	0.37 ± 0.02^{a}	24.69 ± 3.27^{a}	14.81 ± 1.19^{a}

All values are mean ± standard deviation of three replicates.
[a-e] Means in the same column (variants and storage time) with different lowercase letters are significantly different ($p<0.05$).
1Control: meat batter without antioxidants; BHA: meat batter with 0.02% BHA ; T1: meat batter with 0.02% of tara powder; T2: meat batter with 0.04% of tara powder; T3: meat batter with 0.08% of tara powder.

The findings observed by TPA were confirmed by the sensory evaluation, where significant differences among cooked pork batters textural properties were identified. Estevez et al. (2006) also reported that the addition of natural antioxidants may enhance texture characteristics of emulsion type meat products by reducing hardness, adhesiveness, gumminess and chewiness. Hayes et al (2011) found that the application of lipid-soluble ingredients such as ellagic acid protected the muscle membrane from lipid oxidation and therefore reduced moisture loss which in turn would have an effect on the sausages textural properties.

5.4 Conclusions

The results presented here suggest that tara (*Caesalpinia* spinosa) pod powder is a potential source of natural antioxidant and can be successfully used to decrease lipid oxidation and improve the shelf life and colour stability of cooked pork products in meat industry. In addition, from a nutritional point of view, the addition of natural functional ingredient to cooked pork products could provide bioactive compounds (polyphenols) and also addresses consumer demands for healthier functional food products.

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6 Antimicrobial activity of tara (*C. spinosa)* pod extracts

6.1 Introduction

Tannins are common secondary metabolites in vascular plants and they are mainly found in leaves, buds, seeds, roots, and tissues. Due to their antimicrobial activity, their main functions include plant defence against many pathogen attacks, and also against from herbivorous animals, making assimilation of substance contained in the plants difficult, and giving them an unpleasant taste. Useful tannic compounds were found in the gall of walnut-tree, in the bark of trees, such as chestnut and oak, in pomegranate (*Punica granatum* L.) and in tara (*Casalpinia spinosa*) pods (Romani, Campo, & Pinelli, 2012). *C. spinosa* known as tara, is a leguminous native to Peru and, due to its antibiotic effects, widely used in traditional medicine since pre-Hispanic times to fight respiratory illness and skin infection (Aguilar-Galvez et al., 2014). The fruit of tara contains approximately 65% of pods and 32-38% of seeds. From the pods, tara powder is obtained by simple mechanical milling and sifting of the gross powder. It has been reported that 40-65% of the fruit mass of *C. spinosa* corresponds to gallotannins. The principal components of tara tannins are based on a galloylated quinic acid structure (Fig. 6.1). Thus, they differ from hydrolysable tannins based upon a galloylated or ellagoylated hexose. In galloylquinic acids, not only may gallic acid moieties be linked to each of the four hydroxyls of the quinic acid but these may form aryl esters with one or more addictional gallic acid moieties (Aouf et al., 2014).

Tannins in general are phenolic compounds with astringent, antiviral, antibacterial, antiparasitic, and antioxidant properties (Arapitsas, 2012). The effect of polyphenols on the growth of bacteria depends on the bacterial strain, phenolic structures and the dosage assayed (Almajano, Carbo, Jimenez, & Gordon, 2008). Extensive studies have been done on the antimicrobial property of polyphenols against the pathogenic intestinal bacteria, but very few studies have been carried out on the effect of polyphenols on beneficial intestinal microflora (China et al., 2012).

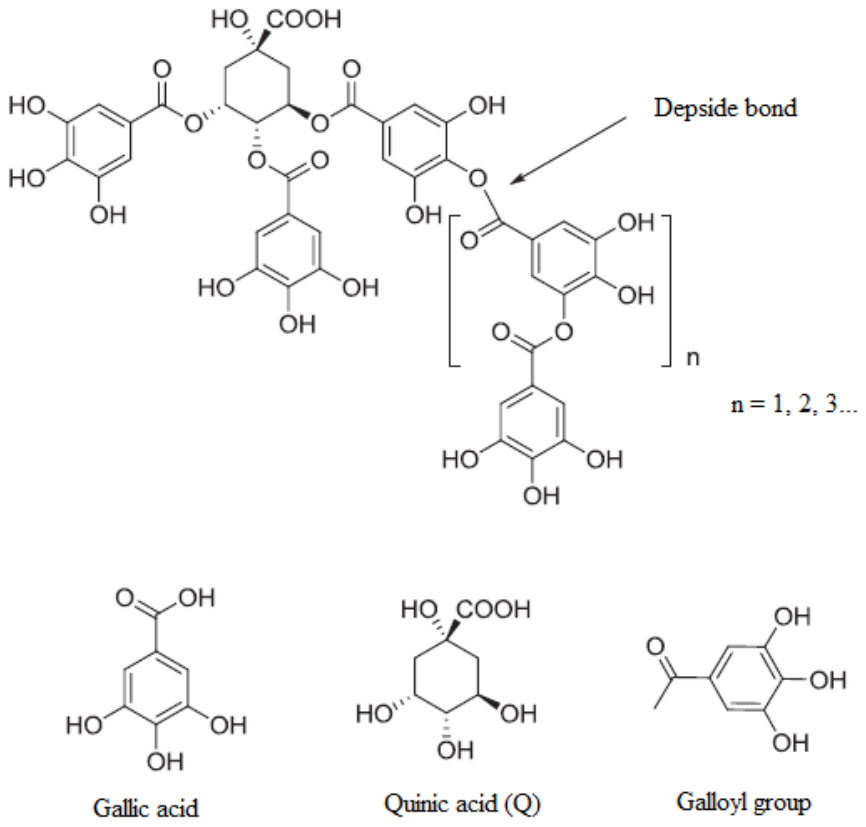

Figure 6.1 Supposed tara tannins chemical structure (Aouf et al., 2014).

The appropriate composition of the intestinal microflora together with the gut immune system allows resident bacteria to exert a protective function. Three microflora modulation tools have emerged: the addition of exogenous live microorganisms to foods (probiotics), the selective stimulation of the growth and activity of beneficial microorganisms indigenous to the gut (prebiotics), and a combination of both approaches (synbiotics). All three approaches attempt to increase the number of bacteria favourable human gastrointestinal health, usually the *Bifidobacterium* and *Lactobacillus* family. Probiotics have been extensively studied and explored commercially in many different products, including dairy and non-dairy products. Probiotic microorganisms contribute to intestinal balance, play a role in maintaining health and can even improve the quality of some products formulated with them (China et al., 2012; Soccol et al., 2010).

Polyphenols (commonly reported as a class of compounds with antibacterial properties) have recently been showed to generate a prebiotic effect (Fogliano et al., 2011).

Phytochemicals and their derivatives can affect the intestinal ecology, as a significant part of them are not fully absorbed and are metabolized in the liver (Montella et al., 2013). Bialonska et al. (2009) analyzed the effect of a commercial extract of pomegranate tannin at 0.01% as well as the effect of its main constituents (0.05%) on the growth of several intestinal bacteria by liquid culturing. A strong inhibition capacity was observed with punicalagins and ellagic acid, especially against *Clostridium* species, while a repression in pathogenic *Staphyloccocus aureus* growth was only obtained with the pomegranate extract and punicalagins. Interestingly, the growth of probiotic *Lactobacillus* and *Bifidobacterium* species was less affected. Moreover, the same group aimed to prove whether this trend was maintained using a fermentation batch-culture system inoculated with fecal samples from healthy individuals, which better simulate conditions in the colonic region. In this experiment, pomegranate extract was able to produce an increment in total bacterial number, enhancing the growth of *Bifidobacterium* spp., *Lactobaciullus* and *Enterococcus* group (Bialonska et al., 2010).

In this study, we evaluated the antimicrobial activity of tara pod extracts against the common human and food-borne pathogenic microorganisms: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Enteroccocus faecalis, Shigella sonnei, Acinetobacter* sp*, Proteus mirabilis, Serratia marcencens, Micrococcus luteus, Apergillus brasiliensis,* and *Candida albicans.* In addition, we studied the effect of the tara pod extracts on the growth of a typical intestinal probiotic bacterium, namely *Lactobacillus plantarum*.

6.2 Materials and methods

6.2.1 Plant material and preparation of the extracts

Fruit pods of *Caesalpinia spinosa* (tara) were commercial product from Peru (Mabrata Tara Powder, Agrotara SAC). Tara pod powder (0.5 g) was extracted with 5 mL of 10% methanol. After 30 min of extraction with magnetic stirring (Multipoint Magnetic Stirer, SBS) at 900 rpm, the sample was held for 30 min in an ultrasonic bath (Prolabo brand equipment) and the extract was separated from the residue by centrifugation at 1900 x g (Sigma 3K30, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany).

6.2.2 Microorganisms and culture conditions

The antimicrobial activity of the tara extract was evaluated against the following strains of microorganisms obtained from the Spanish Type Culture Collection (Valencia, Spain):
– gram-positive bacterial strains: *Staphylococcus aureus* CECT 239, *Entrococcus faecalis* CECT 795, *Lactobacillus plantarum* CECT 4308, *Micrococcus luteus* CECT 245
– gram-negative: *Escherichia coli* CECT 516, *Pseudmonas aeruginosa* CECT 111, *Salmonella enterica* CECT 4155, *Shigella sonnei* CECT 4631, *Acinetobacter* sp CECT 5798, *Proteus mirabilis* CECT 170, *Serratia marcencens* CECT 159
– fungus *Aspergillus brasiliensis* CECT 2574 and
– yeast *Candida albicans* CECT 1394.

All bacteria except *Lactobacillus* were harvesting in Tryptone Soya Agar (TSA) (Oxoid, UK) agar plates at 37ºC and were incubated overnight (o/n). *Lactobacillus* was harvesting in de Man, Rogosa, and Sharpe (MRS) (Oxoid, UK) agar at 37 ºC o/n in a modified atmosphere with 5% of CO_2. *C. albicans* was harvesting in saboraud-chloramphenicol (SB-Cl) (Oxoid, UK) agar medium at 30ºC for 48h and *A.brasiliensis* was harvesting as well in Sb-cl agar medium and incubated at RT for 4 days.
All experiments on antimicrobial activity of tara extracts were conducted three times independently. In addition, before all experiments the sterility of tara extracts was checked by harvesting in TSA.

6.2.3 Disc diffusion assay

The antimicrobial activity of tara extracts was evaluated by the disc diffusion method (Sharififar et al., 2007). Active cultures used for experiments were incubated in triptone soy broth (TSB, Scharlab, S.L., Barcelona, Spain) overnight. Cell density was optically determined at 620 nm in a UV-Vis mini 1240 Shimadzu spectrophotometer in comparison with previous calibration curves with same microorganism. Inoculum was prepared by dilution to achieve cell densities of 10^4 CFU/mL in Ringer (Biokar Diagnostic, Beauvais, France). Inoculum (10-500 µL) was mixed with 10 mL of the growth media (TSA, MRS, RCM or SB-Cl) and was spread on agar plate. Tara extract (30 µL) was added to 6 mm diameter discs. Plates were incubated for 24h, 48 h or 4-5 days (depending on the type of microorganism) and antimicrobial activity was evaluated by measuring the inhibition zone against microorganisms. Assays were performed in triplicate.

6.2.4 Minimum inhibitory concentration assay

Minimum inhibitory concentration (MIC) was determined in ninety-six-well plates by the method of successive dilutions as described by Delaquis et al. (2002) with some modifications. Media (150 µL) was added to wells containing 50 µL of an appropriate dilution of the tara extracts and 5 µL of overnight and direct inoculum at 10^4 CFU/mL (*S. aureus* and *L. plantarum*) and incubated at 37 °C for 24 h. Concentrations tested ranged from 0.20 to 25 mg/mL of tara extract. The growth of bacteria in treatment wells was compared to controls (media + inoculum; media + extract). The absorbance was measured at 620 nm using an absorbance microplate reader ELISA ELX80 (BioTek, Vermont, USA). MIC was the dilution at which growth was not observed.

In order to study the growth-promoting effect of tara pod extract on *Lactobacillus plantarum*, the same *in vitro* methodology was used as in the MIC assay (see above). Concentrations tested in the ninety-ssix-well plates ranged from 0.20 to 25 mg/mL. The tara pod extract concentration corresponding to maximum probiotic growth was determined (MGC – maximum growth concentration). Assays were performed in triplicate.

6.3 Results and discussion

The results are presented in Table 6.1. When comparing all the microorganisms studied, it becomes clear that the strain *S. aureus* is the most sensitive, showing the highest inhibition diameter in the presence of the tara pod extract. The second most sensitive strain was *Shigella sonnei*, followed by *M. luteus* and *P. mirabilis*.

Among all the bacteria strains tested *S. aureus* showed the biggest inhibition halo and for this reason this strain was selected to assess the MIC. The value found was 6.25 mg/mL of tara pod extract. Aguilar-Galvez et al. (2014) also reported that tara pod extracts or the products of their acid hydrolysis inhibited the growth of several bacterial strains including *Bacillus subtilis, Micrococcus luteus, Listeria innocua* and *Staphylococcus aureus.* In addition, *E. coli* was found to be the most resistant to tara pod extracts and no antifungal activity was observed, which is in agreement with previous studies (Kloucek et al., 2005).

Table 6.1 Antimicrobial activity of tara pod extract.

Strain	Disc inhibition zone (mm)	MIC[a] or MGC[b] (mg/mL)
Staphylococcus aureus	16.0 ± 0.1	[a]6.25 ± 0.0
Entrococcus faecalis	NA	-
Lactobacillus plantarum	*	[b]0.40 ± 0.0
Micrococcus luteus	6.5 ± 0.2	-
Escherichia coli	NA	-
Pseudmona aeruginosa	NA	-
Salmonella enterica	NA	-
Shigella sonnei	12.2 ± 0.8	-
Acinetobacter sp	8 ± 0.5	-
Proteus mirabilis	6.5 ± 0.4	-
Serratia marcencens	NA	-
Aspergillus brasiliensis	NA	-
Candida albicans	NA	-

Disc inhibition zone expressed as the mean ± SD in mm
MIC: minimal inhibitory concentration expressed as the mean ± SD in mg/mL of tara pod extract;
MGC: maximum growth concentration expressed as the mean ± SD in mg/mL of tara pod extract;
NA: not active; ¨-¨ not performed; * growth stiumulating effect on the probiotic strain

On the other hand, tara pod extract showed growth promoting effect on the common probiotic bacterium *L. plantarum* with MGC value of 0.40 mg/mL. Several studies have shown that polyphenolic compounds including tannins may stimulate the growth of intestinal microorganisms, suggesting that they also could be potential prebiotic molecules. Khalil (2010) reported the influence of gallic acid and catechin polyphenols on growth and some recently proven probiotic attributes of *Streptococcus thermophiles.* Vodnar and Socaciu (2012) found that green tea coencapsulated with *B. infantis* and *B. breve* exert a protective effect on bacteria during exposure to gastrointestinal conditions and refrigerated storage. Another research group reported that resveratrol, a potent antioxidant found in wine, promotes the growth of *Biffidobacterium* spp. and *Lactobacillus* spp. (Larrosa et al., 2009). Polyphenols may also significantly affect intestinal bacterial population by decreasing the pH of the intestinal environment. Generally, lower pH favors probiotic bacteria as compared to pathogenic bacteria, which are highly variable in their tolerance to acids (Bialonska et al., 2009).

6.4 Conclusions

This study provides strong evidence that a polyphenolic compound interaction with *L. plantarum* may stimulate the prebiotic properties of functional food products which are

gaining interest and have recently been introduced into the market. The products of this kind (such as yogurt, vegetable juice or meat products) would acquire additional value if the phenolic compounds could protect probiotic strains during down-stream processing, formulation and/or storage, thus improving their viability and stability. Therefore, the influence of tara pod extracts on individual bacteria strain observed in the present *in vitro* study should be verified by additional research under conditions resembling the actual probiotic food production. In addition, the growth-promoting effect seems to be strain-dependent (Gaudreau et al., 2013) and in the future work other common probiotic strains used in functional foods should be taken into consideration.

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7 The effect of *Perilla frutescens* extract on the oxidative stability of model food emulsions

7.1 Introduction

In most foodstuffs, lipid oxidation is a severe problem that causes rancid odors and flavors, modifies texture and color and decreases shelf life (Alamed, Chaiyasit, McClements, & Decker, 2009). These changes degrade functional and nutritional compounds of food, damage essential fatty acids and produce oxidized polymers which could raise safety concerns. Especially, this process is favored in oil-in-water emulsions because of the large contact surface between the oxidizable lipid hydroperoxides in emulsion droplets and water-soluble prooxidants resulting in the propagation of oxidation reactions (Waraho et al., 2012). To avoid this problem, synthetic antioxidants are commonly used, such as butylated hydroxytoluene and butylated hydroxyanisole (Brewer, 2011). However, in recent years consumers have become increasingly concerned about the impact of food and food ingredients on their own health, and this attitude has caused several changes throughout the food industry (Falguera, Aliguer, & Falguera, 2012). As part of these changes, food companies have been forced to seek for natural-origin counterparts for a number of ingredients that fulfill technological functions (Skowyra, Falguera, Gallego, Peiró, & Almajano, 2013).

Plant extracts rich in phenolic compounds may be a good alternative to synthetic antioxidants to prevent lipid oxidation. Plants produce phenolic compounds to deal with reactive oxygen species and free radicals produced during photosynthesis. Inside the plant structure, lipid peroxidation has been known to associate with tissue injuries and disease conditions. Plant phenolic compounds can act as protective factors delaying the onset of lipid oxidation and decomposition of hydroperoxides in living tissues (Wettasinghe & Shahidi, 1999). Therefore, such compounds are expected to play a similar role inside food matrices, provided that a suitable extraction and conservation method is found and optimized. In the process of testing new plant extracts as antioxidants, two major difficulties arise. On the one hand, there are several factors in the extract-obtaining process that must be analyzed, the most important of which are the method and the solvent used. These factors will determine the antioxidant content and activity of the resulting mixtures (Santas, Carbo, Gordon, & Almajano, 2008). On the other hand, very

often the results obtained when studying the isolated products using the *in vitro* tests correlate poorly with their ability to avoid oxidative impairment of foods (Decker et al., 2005, Ramful et al., 2011). This lack of equivalence is the consequence of complex interactions among the components of the food matrix, which may limit or enhance the activity of the tested extract. Therefore, functionality tests must be performed in model foods that consider the most important conditions of the actual food in which they are to be applied. In this way, oil-in-water emulsions have become a standardized model food to test the protective effect of antioxidant products against lipid oxidation (Almajano & Gordon, 2004). Phenolic extracts of certain plant materials have been shown to neutralize free radicals in model systems. Widely used culinary herbs of the *Lamiaceae* family such as rosemary, thyme, marjoram and oregano have gained the interest of many research groups (Al-Bandek et al., 2011, Gallego et al., 2013, Poyato et al., 2013).

Perilla frutescens (*Lamiaceae* family) is a traditional Chinese medicinal plant that is commonly used for a variety of diseases such as depression, inflammation, bacterial and fungal infections, allergy, intoxication, some intestinal disorders and even tumors (Lin et al., 2010; Mao et al., 2010). In Asian countries such as Japan, Korea and China its leaves are commonly added to sushi, garnishes and soups, and young raw leaves are often used to wrap cooked food. Its health-promoting effects have been mainly attributed to its content of phenolic acids (e.g. rosmarinic acid), flavonoids and triterpenoids (Hong & Kim, 2010; Lee et al., 2013). These components provide the extracts of this plant with proved antioxidant, anti-inflammatory, antibiotic and antipyretic properties. Moreover, *Perilla frutescens* seed oil (known as PFSO) has also been shown to be a rich source of unsaturated fatty acids, especially omega-3 linolenic acid (Eckert et al., 2010). Although not yet reported in the literature, perilla extracts, being a rich source of various phenolic compounds could therefore be incorporated in model emulsions as a source of natural antioxidant to prolong quality and stability.

The aim of this paper is to report a study of the antioxidant properties of purple perilla (*Perilla frutescens*) extracts in model emulsions stored for long periods, which can be representative of real food systems and their expected shelf life. Lipid oxidation was determined by following the formation of peroxide values as the primary oxidation products and thiobarbituric acid reactive substances and hexanal content as the secondary products. In addition, the content of the main phenolic compounds in perilla cultivated in Spain has been quantified.

7.2 Materials and Methods

7.2.1 Raw material

Purple perilla was grown in a greenhouse (Balaguer, Spain). Stalks and leaves of perilla were collected, dried and ground to a homogenous powder in collaboration with the company *Pàmies Hortícoles*. The powder was stored in darkness, at room temperature until extraction. Refined sunflower oil was purchased in a local market.

7.2.2 Reagents

6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, rosmarinic acid, caffeic acid, phosphate buffered saline (PBS), 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), 2,2,-azobis-(2-methylpropionamide) dihydrochloride (AAPH), fluorescein ($C_{20}H_10Na_2O_5$) and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK). Folin-Ciocalteu reagent, absolute ethanol, aluminum oxide, ferric chloride ($FeCl_3$), ammonium thiocyanate (NH_4SCN), anhydrous sodium carbonate and Tween 20 were analytical grade from Panreac (Barcelona, Spain).

7.2.3 Extraction

Air-dried and finely ground perilla was weighed (4 g) and extracted with 60 mL of ethanol-water mixture at 50:50 (v/v). The mixture was stirred continuously for 24 h at 4°C. After that, all samples were centrifuged (Sigma 6K10, Germany). Part of the supernatant was used to determine the antiradical capacity. The volume of the remaining supernatant was measured and the solution was evaporated, frozen at -80°C for 24 hours and lyophilized for 3 days. Samples were then weighed and kept protected from light in a desiccator until used to prepare an oil-in-water emulsion system.

7.2.4 Total phenol and flavonoid content

Total polyphenol content (TPC) of extracts was determined by colorimetric spectrophotometry following the Folin-Ciocalteu method (1999), slightly modified and adapted for microplates. Samples were taken from the extract solutions, diluted 1:30 (v:v) and Folin-Ciocalteu reagent (4% by volume), 20% sodium carbonate solution (30.8% by volume) and Milli-Q water were added. Samples were well mixed and left in the dark for 1 h. The absorbance was measured at 725 nm using a UV–vis spectrophotometer

(Fluostrar Omega, Perkin-Elmer, Paris, France) and the results were expressed in gallic acid equivalents, GAE, using a gallic acid standard curve (10-70 µM).

Total flavonoid content (TFC) of perilla extracts was measured according to the method of Zhishen et al. (1999). Each sample (500 µL) was mixed with 5% $NaNO_2$ (75 µL), and then left to stand for 5 min at room temperature. The mixture was sequentially mixed with 150 µL 10% $AlCl_3$, 500 µL 1 M NaOH and 275 µL distilled water. The absorbance at 510 nm was measured using spectrophotometer UV-4201/20 (Zuzi, AuxiLab, S.L., Spain). Values were determined from a calibration curve prepared with catechin (ranging from 6 to 60 mg/L) and expressed as mg of catechin equivalent per gram of dry/fresh weight (CE/g DW/FW).

7.2.5 Antioxidant capacity determination

7.2.5.1 ABTS assay

The first method used was the 2,2'-azinobis-(3-ethyl-benzo-thiazoline-6-sulfonic acid (ABTS) discoloration assay (Re et al., 1999). The assay is based on the ability of an antioxidative compound to quench the $ABTS^+$ radical relative to that of a reference antioxidant such as Trolox. A stock solution of ABTS radical cation was prepared by mixing ABTS solution with a potassium persulfate solution at 7 mM and 2.45 mM final concentration, respectively. The mixture was maintained in the dark at room temperature for 16 h before use. The working $ABTS^+$ solution was produced by dilution of the stock solution in 10 mM PBS (pH 7.4) incubated at 30°C to achieve an absorbance value of 0.7 (±0.02) at 734 nm. An aliquot of 20 µL of diluted extract was added to $ABTS^+$ radical working solution (180 µL). For the blank and standard curve, 20µL of PBS or Trolox solution were used, respectively. Absorbance was measured by means of a UV–Vis spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) at 734 nm and percent inhibition was calculated as Skowyra et al. [5]. The radical-scavenging capacity of extracts was quantified as mg of Trolox equivalent per gram of dry/fresh weight.

7.2.5.2 The oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) method was adapted from Ou et al. (2001). The assay was performed with an automated microplate reader and 96-well plates. Diluted extract (40µL) was transferred by pipette into each well and then 120 µL of 1.34 µM fluorescein working solution in phosphate buffer (13.3 mM) at 37ºC were added to

each sample. The plate was placed in a spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) and incubated at 37ºC. The initial fluorescence was recorded at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. 2,2`-Azobis(2-metylpropionamide) dihydrochloride (AAPH, 40 µL, 30 mM) was then added to each sample well and the fluorescence was measured immediately and every 2 min thereafter for 120 min. For the calibration curve, solutions of Trolox were prepared in the range of 8-58 µM. The ORAC value for each extract was calculated using a regression equation relating Trolox concentration and the net area under the fluorescence decay curve. Results are expressed as mg of Trolox equivalents per gram of dry/fresh weight.

7.2.5.3 FRAP assay

The FRAP assay was performed as described by Benzie & Strain (1996) with some modifications. The FRAP reagent was prepared with acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl, 40 mM) and $FeCl_3$ (20 mM). The proportions were 10:1:1 (v:v:v), respectively. A suitable dilution of the extract was added to the FRAP reagent (1:30, v:v) and incubated at 37ºC. The assay was performed by means of an automated microplate reader (Fluostar Omega, Perkin-Elmer, Paris, France) with 96-well plates. The absorbance at 593 nm at time zero and after 4 min was recorded. The analysis was performed in triplicate and values were determined from a calibration curve of Trolox (ranging from 2.5 to 33 µM). The results are expressed as mg of Trolox equivalent per gram of dry/fresh weight.

7.2.6 Determination of cinnamic acid derivatives by high-performance liquid chromatography (HPLC)

HPLC analyses of the perilla extracts were carried out using an Acquity UPLC System (Waters, USA) with photodiode array (PDA) detector. Perilla extracts (10 µL) were injected onto an analytical C_{18} column (Symmetry, 5 µm, 3.9 x 150 mm, Waters) at 25°C. The mobile phase was composed of 0.5% formic acid (v/v) in acetonitrile (eluent A) and 0.5% formic acid (v/v) in water (eluent B). The gradient program was as follows: 10% A (20 min), 35% A (4 min), 10% A (6 min). Total run time was 30 min. The absorbance at 330 nm was measured to detect the cinnamic acid derivatives (caffeic acid and rosmarinic acid). The standard was identified by its retention time and its concentration was calculated by comparing the peak area of samples with that of the standard. Standard solutions with concentrations ranging from 10 to 100 ppm were then prepared by diluting

the stock standard solution with water. The perilla extracts were filtered through a 0.45 µm filter for HPLC analysis.

7.2.7 Oil- in-water emulsion system

7.2.7.1 Removal of tocopherols from sunflower oil

Tocopherols were removed from sunflower oil by column chromatography using activated alumina, as described by Yoshida et al. (1993). The oil was stored at -80 ºC prior to emulsion preparation (up to 2 days). The fatty acid composition of the filtered sunflower oil is shown in Table 7.1. The fatty acid composition was determined using a method based on that of Conde et al. (2011). The sunflower oil used contained linoleic acid (52.38%) and oleic acid (34.51%) as the main unsaturated fatty acids.

Table 7.1 Fatty acid composition of sunflower oil.

Fatty acid name	Numerical symbol	Amount (%)
Saturated		**12.79**
Palmitic acid	C16:0	6.99 ± 0.08
Stearic acid	C18:0	4.16 ± 0.04
Arachidic acid	C20:0	0.33 ± 0.01
Behenic acid	C22:0	0.96 ± 0.02
Lignoceric acid	C24:0	0.35 ± 0.02
Unsaturated		**87.21**
Oleic acid	C18:1 (*n*-9)	34.51 ± 0.11
Eicosenoic acid	C20:1 (*n*-9)	0.32 ± 0.03
Linolenic acid	C18:2 (*n*-6)	52.38 ± 0.23

7.2.7.2 Preparation of emulsions and storage conditions

Oil-in-water emulsions were prepared with 1% of Tween 20 as emulsifier and 10% of sunflower oil. Emulsions were prepared by dropwise addition of oil to the water phase, with sonication using a UP200S ultrasonic (Hielscher Ultrasonics GmbH, Germany) during cooling in an ice bath for 10 min. It was necessary to repeat sonication 7 times (7 x 10 minutes) to have enough volume of emulsion. Freeze-dried powder of the perilla extract was redissolved in ethanol 50% (v/v) and added directly to the emulsion and homogenized, obtaining final concentrations of 80 and 320 ppm (C1 and C2, respectively). For the negative control no extract was added, and the positive controls were prepared with Trolox (40 ppm) and BHA (20 ppm) dissolved in ethanol.

All emulsions were stored in triplicate in 60 mL amber bottles in the dark, with constant elliptical movement and allowed to oxidize at 32 ± 1 ºC for 30 days.

7.2.7.3 Measurement of primary oxidation by peroxide value (PV) and pH

Peroxide value (PV) was measured periodically (every 2 or 3 days the time of storage) using aliquots of 0.05–0.1 g of each sample and determined by the ferric thiocyanate method (Frankel, 1998), after calibrating the procedure with a series of oxidized oil samples analyzed by the AOCS Official Method Cd 8-53 (1997).

The pH of the samples was measured (pH-meter GLP21, Criston Instruments, Spain) as a parameter to investigate its correlation with PV.

7.2.7.4 Measurement of secondary oxidation by TBARs and hexanal methods

The thiobarbituric acid reactive substances (TBARs) assay was performed as described by Maqsood & Benjakul (2010) with some modifications. One mililiter of oil-in-water emulsion sample was mixed with a TBARs solution containing 0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl solution (5 mL). The samples were placed immediately in an ultrasonic bath (Prolabo brand equipment) for 5 min and heated in a water bath (95ºC) for 10 min. The mixture was centrifuged (Sigma 3K30, Sigma Laborzentifugen GmbH, Germany) at room temperature at 4000 rpm for 10 min. The absorbance of the supernatants was measured at 532 nm (Spectrophotomter UV-4201/20, Zuzi, Spain). The TBARs values were expressed as mg of malondialdehyde (MDA) per kg of emulsion calculated using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, USA) as the standard.

Hexanal was measured according to Waraho et al. (2012) with some modifications using a TRACE gas chromatography equipped with MS DSQI (ThermoFisher Scientific) and TRIPLUS auto-injector. One mililiter of emulsion was weighted into a head space vial and equilibrated at 60°C for 30 min. Aliquots (1 mL) of the head space were injected onto a DB-624 column (60 m x 0.32 mm x 1.8 µm). Initially, the oven temperature was set to 60°C, maintained at this value for 2 min, then raised up to 220°C at 8°C/min and maintained for 5 min. The injection port was operated in the split mode. The carrier gas was helium at flow rate of 1.8 mL/min. A flame ionization detector was used at a temperature of 260°C. Hexanal concentrations were determined from peak areas using a standard curve prepared with hexanal standard solutions.

7.2.8 Statistical analysis

TPC, TFC, $ABTS^{+}$, ORAC and FRAP measurements were performed in triplicate on triplicate samples. PV, TBARs and hexanal measurements were performed once on triplicate samples.

Mean values for different parameters were calculated and compared by analysis of variance (one-way ANOVA) using commercial software (Minitab 16). Moreover, statistical differences between mean values were identified at the 95% of confidence level ($p<0.05$). Person's correlation analysis was performed using the same statistical package.

7.3 Results and Discussion

7.3.1 Phenolic and flavonoid content of extract

The total polyphenols and flavonoids in the ethanolic extracts of *P. frutescens* leaves and stalks are shown in Table 7.2. The perilla extract contained 22.67 ± 0.52 mg gallic acid equivalent (GAE)/g DW and 2.90 ± 0.07 mg catechin equivalent (CE)/g DW. Hong and Kim (2010) reported a similar content in perilla leaves (12.15 mg GAE/g DW and 7.23 mg rutin equivalent (RE)/g DW, respectively) using 70% ethanol in refluxed extraction for 24 h. Similarly, Kee et al. (2013) found a value of 27.10 mg GAE/g DW after aqueous extraction. In addition, studies involving methanol extraction of perilla leaves have reported lowest values than those obtained in the present study, in the range of 0.7-1.1 g GAE/100 g fresh weight (FW), using a mixture of water-methanol-formic acid (15:80:5) (Müller-Waldeck, Sitzmann, Schnitzler, & Grassmann, 2010). Lin et al. (2010) also studied the extraction of phenolics and flavonoids from perilla leaves and stalks separately and the methanolic extract of stalk had higher polyphenol and flavonoids content than that of leaves (137.40 mg GAE/L and 205.75 mg RE/L, respectively). Consequently, the extraction method and the solvent used play a key role in the extraction of polyphenols and flavonoids from plant material. Likewise, Hong et al. (2011) studied phenolic-enriched fraction from *P. frutescens* and the highest total phenolic and flavonoid level was detected in the ethyl acetate fraction from the water extract (373.92 mg GAE/g fraction and 86.63 mg RE/g fraction, respectively). Whatever the considered extraction method or solvent, perilla was shown to have polyphenol level like typical culinary herbs from the same family (*Lamiaceae*) such as basil (Hossain et al., 2011), spearmint (Kee et al., 2013), marjoram and salvia (Tawaha et al., 2007).

Table 7.2 Polyphenol and flavonoid content and antioxidant activity of perilla extract.

Method	Dry weight	Fresh weight
Total phenol content (mg GAE/g)	22.67 ± 0.52	34.00 ± 0.78
Total flavonoid content (mg CE/g)	2.90 ± 0.07	4.35 ± 0.10
ABTS (mg TE/g)	65.03 ± 2.98	97.54 ± 4.47
ORAC (mg TE/g)	179.60 ± 6.02	269.40 ± 9.03
FRAP (mg TE/g)	44.46 ± 1.55	66.70 ± 2.32

Values are mean ± standard deviation (n=3).

7.3.2 In-vitro antioxidant activity of extract

Antioxidant activity of the extract from *P. frutescens* was assessed by three different methods: $ABTS^+$, ORAC and FRAP (Table 7.2). The use of several methods provides more comprehensive information about the antioxidant properties of the original product (Martínez et al., 2012). An $ABTS^+$ value of 65.03 ± 2.98 mg TE/g DW, an ORAC value of 179.69 mg TE/g DW and a FRAP value of 44.46 mg TE/g DW were measured in the ethanolic leaf and stalk extract. Similarly, Muller-Waldeck (2010) found the $ABTS^+$ value of 0.8-1.3 g TE/100 g fresh weight (FW) in perilla leaves using 80% methanol with 5% formic acid. Lin et al. (2010) reported that a scavenging abilities of the methanolic extracts of stalk and leaf from *P. frutescens* at 1.5-25 µg/ml on DPPH radicals were in the range of 18.7-91.0% and 6.7-63.1%, respectively. Meng et al. (2009) also found an activity of 114-167 µmol TE/100 mL of aqueous perilla extract as assessed via DPPH assay. Hong et al. (2011) described antiradical activity (DPPH) and reducing power of the phenolic-enriched fractions from *P. frutescens*, finding strong reducing power and effective radical scavenging activity (89.48-90.74%) of the ethyl acetate fractions of methanolic extracts. They also reported the antioxidant activity (in β-carotene/linoleic acid system) of the phenolic-enriched fractions from perilla leaves, finding that at 500 µg/ml chloroform fractions of 70% ethanol extract presented remarkable antioxidant abilities in the linoleic acid emulsion system. Tawaha et al. (2007) assessed the antioxidant activity of selected plant species such as rosemary, marjoram and salvia by $ABTS^+$ method, obtaining in all cases lower values (of the order of µmol TE/g DW) than those found for perilla in this study. Also Hossain et al. (2011) reported that the ORAC values for basil and parsley were 17.57 and 13.25 g TE/100 g DW, respectively. This value is much lower than those found in the current study.

7.3.3 Quantitative analysis of cinnamic acid derivatives

Lately, phenolic compounds, such as rosmarinic acid or caffeic acid have aroused increasing interest due to their antioxidant activity, which improves the stability of lipid-containing foods (Conde et al., 2011; Medina et al., 2012) and their possible beneficial effects on human health (Yang, Hong, Lee, Kim, & Lee, 2013). The concentrations of rosmarinic acid (2.29 ± 0.09 mg/g DW) and caffeic acid (0.51 ± 0.02 mg/g DW) were compared with those reported in the literature (Figure 7.1, Table 7.3).

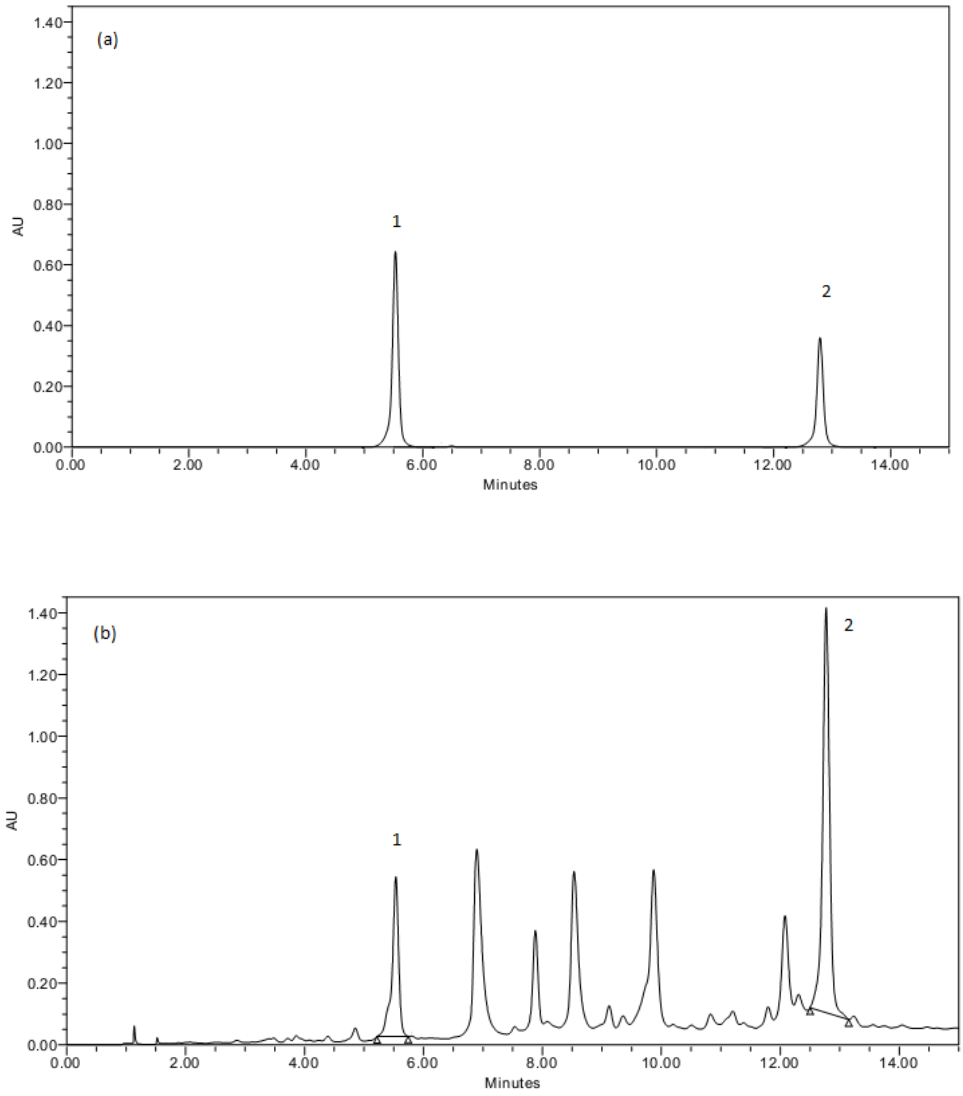

Figure 7.1 Chromatographic profiles, acquired at 330 nm, of perilla ethanolic extract. (a) External standards: 1 - caffeic acid; 2 - rosmarinic acid; (b) Perilla extract.

It is well known that the different extraction conditions lead to different amount of polyphenols in plant extracts. Although many studies have reported the content of cinnamic acid derivatives in *P. frutescens*, researches on the content of the main phenolic compounds in perilla cultivated in Europe has not been measured yet.

Table 7.3 Content of rosmarinic acid and caffeic acid in the perilla extracts (mg/g DW).

Rosmarinic acid	Caffeic acid	Solvent	Place of cultivation	Reference
39.5	ND	Water : acetone : hydrochloric	Japan	Natsume et al.(2006)
3.4 – 10	0.05 - 1.2	Water with 0.01M H_2SO_4	China and Japan	Meng et al.(2006)
0.21 - 3.76	ND	70% EtOH	Geochang, Korea	Hong & Kim(2010)
51.37 - 155.50	ND	MeOH with ethyl acetate fraction	Geochang, Korea	Hong et al.(2011)
29.28 - 54.76	1.09 - 3.86	MeOH with 1% TFA	Yeongnam, Korea	Kang & Lee(2011)
26.84	1.32	Water at 100ºC	Miryang, Korea	Yang et al.(2013)
2.29	0.51	50% EtOH	Spain	This paper

The utilization of perilla opens interesting possibilities for the development of functional foods not only in Asian countries, but also in Europe. Moreover, the content of cinnamic acid derivatives in perilla extract was higher or similar to those reported by Lee (2010) in the dried plants (*Lamiaceae*) such as basil, marjoram, oregano, rosemary or thyme, which are common culinary herbs.

7.3.4 Antioxidant activity of extracts in model emulsion system

Besides their effects on human health upon direct ingestion, one of the main applications of antioxidant extracts is their use in the food industry to avoid or delay oxidation of perishable systems (Kang & Lee, 2011). In this study, this technological ability has been assessed in oil-in-water emulsions as a model food used to test the 50% ethanol extract of perilla. The oxidation was periodically followed by measurement of peroxide value (as indicator of primary oxidation products) and TBARs value and hexanal content (as indicator of secondary oxidation products) during storage at 32±1ºC for 30 days. In addition the change in pH was monitored, since pH tends to fall during oxidation.

Peroxide values in the oil emulsions increased significantly faster in the sample without any antioxidant addition (Fig. 7.2), reaching 10 meq hydroperoxides/kg of emulsion (this value is the allowed limit for products containing edible fats) after 6 days. The next samples to reach this level of deterioration were P_C1 perilla at 80 ppm (after 15 days) and Trolox at 40 ppm (after 19 days). Other samples: P_C2 and BHA were stable until the end of the experiment (after 30 days, PV was < 10 meq/kg). Perilla extract at 320 ppm (P_C2) was as effective as BHA at 20 ppm in preventing the oxidation in emulsions during storage. The mean peroxide value recorded for control emulsions after 24 days, was more than twice the value obtained for emulsions containing the perilla extract at 80 ppm and Trolox at 40 ppm (control: 103.17 meq/kg; perilla C1: 36.55 meq/kg; Trolox: 39.42 meq/kg).

Kiokias et al. (2009) reported peroxide values between 45.60 and 51.15 meq/kg after 2 months in 10% sunflower oil-in-water emulsions with 2g/L of different carotenoids including *β*-carotene, lycopene, paprika, lutein and bixin. Although the time needed to reach these values was remarkably shorter in this study, the concentration of antioxidant extracts was much lower as well (80 and 320 ppm). Kiokias et al. (2008) reported that a 10% cottonseed oil-in-water emulsion with quercetin at 1.5 mmol/kgr took 2.7 days at 60°C to reach a PV of 67.07 meq/kg. Also Maisuthisakul et al. (2006) reported that a 10% oil-in-water emulsion with 100 mg/kg teaw (*Cratoxylum formosum* Dyer) extract took 4.55 days at 60ºC to reach a PV of 50 meq/kg. In this work, the emulsion containing 80 ppm of perilla extract exceeded this value at the 30th day at 32ºC. Ramful et al. (2011) found that *Eugenia pollicina* leaf extract at a concentration of 0.02% was also effective in slowing down hydroperoxide formation in soybean oil emulsion during 13 days of storage at 40ºC. Roedig-Penman et al. (1997) reported that tea extracts added to sunflower oil-in-water emulsion were very effective in their stabilization, the tea extract (0.03%) being similar to BHT (0.02%) and taking 40 days of storage at 30°C to reach a PV of 30 meq/kg. In the same study the rosemary extract (0.03%) in the early stages of storage under these conditions had moderate antioxidant activity up to PV of 20 meq/kg.

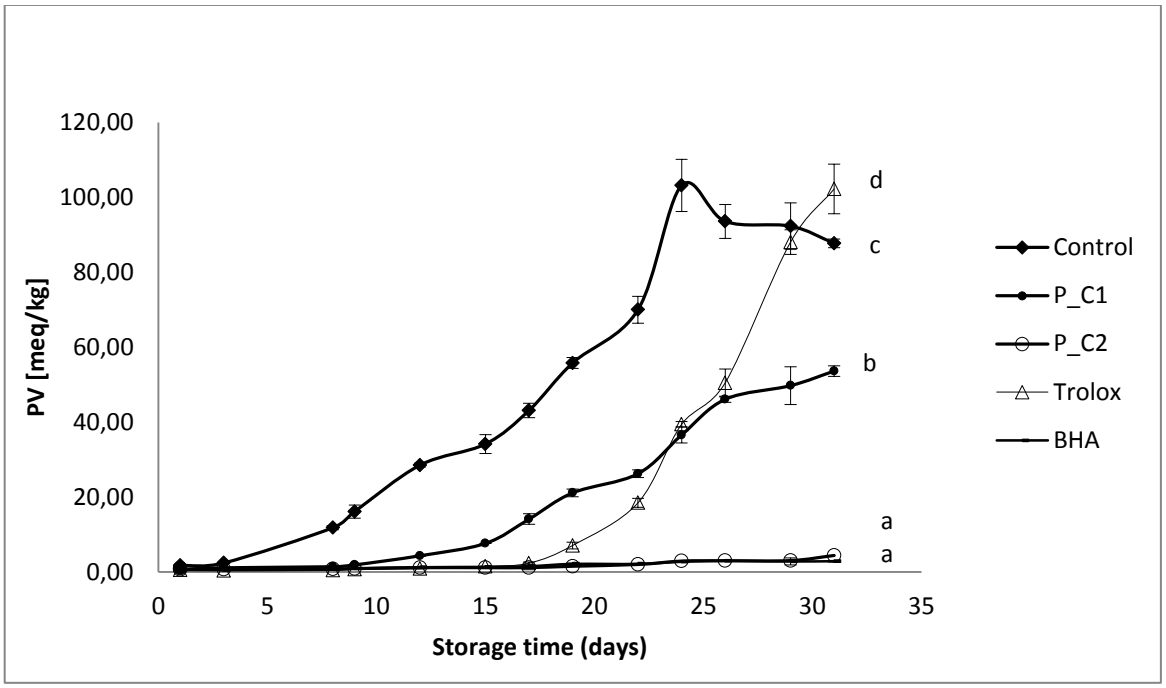

Figure 7.2 Evolution of primary oxidation (peroxide value) in model food system with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).
[a-d] Values with different letters at 30[th] day of storage are significantly different ($p<0.05$).

Hydroperoxides are the main primary products of lipid oxidation, but they are highly unstable and easily break down into secondary compounds, resulting in the appearance of aldehydes, ketones, epoxides or organic acids, which may lead to changes in the pH (Roedig-Penman & Gordon, 1997). In addition, since it is known that many antioxidant molecules are less effective when the pH is low (Sun, Wang, Chen, & Li, 2011), this parameter was also measured as a potential indicator of oil-in-water emulsions oxidation. From an initial average value of 5.12, the samples without any antioxidant addition and with Trolox tended to stabilize their pH at 3.22 and 3.62, respectively after 30 days (Fig. 7.3). In the P_C1, P_C2 and BHA samples the pH slowly increased during storage, but in P_C1 pH it decreased rapidly after 24 days, reaching the value of 4.05. Observing this relationship confirmed that the pH fell as PV increased. Gallego et al. (2013) reported that following the order of primary oxidation, the pH showed a decline (from 6 to 3) proportional to the rate of oxidation in oil-in-water emulsion with 100 ppm of different extracts from rosemary, thyme and lavender. Sorensen et al. (2008) also reported that lipid oxidation increased when pH was decreased from 6 to 3 in a 10% oil-in-water emulsion. Moreover, Zhou et al. (2013) found that the pH appeared to play a significant role in controlling the net antioxidant and pro-oxidant capacity of polyphenols in lipid model systems. The addition of (–)-epigallocatechin-3-gallate (EGCG) at 5-100 μM to

food emulsions was observed to exhibit pro-oxidant activity in low pH (pH 2-4). On the other hand at higher pH values studied (pH 5-7), lower levels of primary and secondary oxidation products were detected in samples with 25-500 μM EGCG.

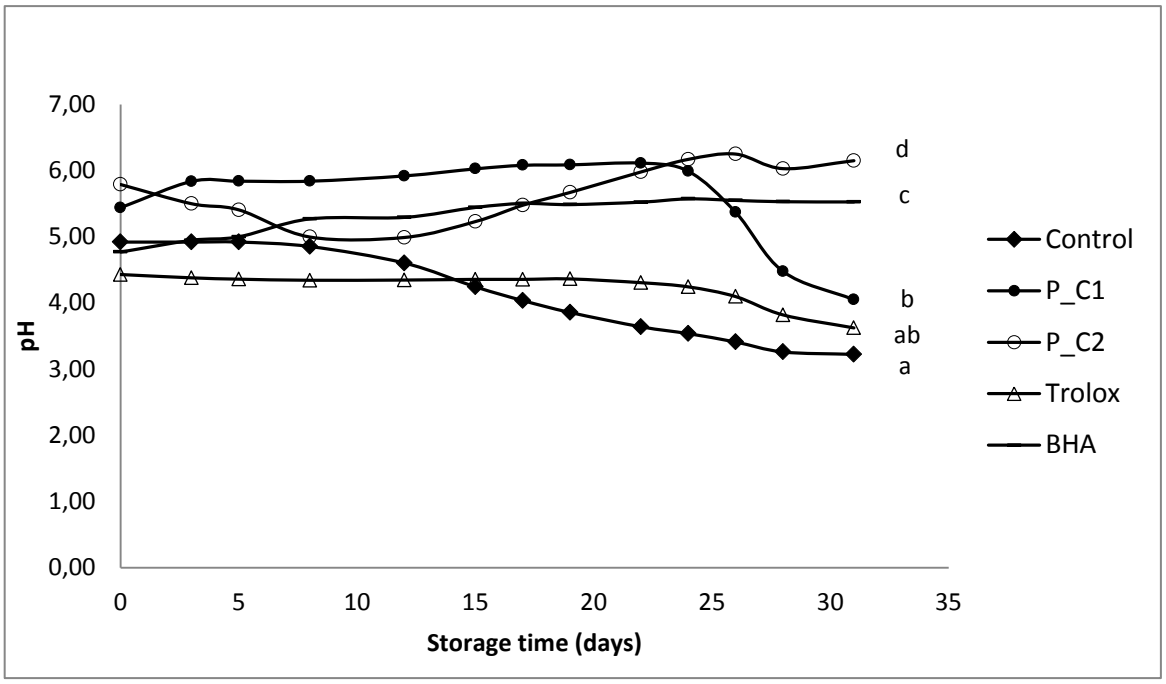

Figure 7.3 Evolution of pH in model food system with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).
[a-d] values with different letters at 30[th] day of storage are significantly different ($p<0.05$).

In the secondary oxidation stage, volatile compounds (e.g. alcohols and aldehydes) are formed by the decomposition of lipid hydroperoxides. In particular, volatile aldehydes have great importance as an indicator of oxidation due to their considerable contribution to the aroma and flavor deterioration of the final product (Conde et al., 2011). Secondary oxidation products in the emulsions were monitored by measurement of the TBARs (Fig. 7.4) and the hexanal content (Fig. 7.5). After 4 weeks, TBARs values of emulsions containing perilla extract and BHA were lower than that of the control (3.82 mg MDA/kg) and the Trolox-containing sample (3.57 mg MDA/kg). BHA was the most effective antioxidant followed by perilla extract P_C2 and P_C1. Garcia-Iñiguez et al. (2010) reported that a lyophilized aqueous extract of *Melissa officinalis* (lemon balm) at 620.6 ppm was as efficient as BHA at 200 ppm in controlling the TBARs formation in oil-in-water emulsions made with a mixture of algae and lineseed oils upon storage during 15 days at 20ºC. Also Poyato et al. reported that in olive oil-in-water emulsions after 48 h of storage at 65ºC, TBARs value was stable and low, with no differences

between lyophilized aqueous extract of lemon balm (477 ppm) and BHA (200 ppm). Dimakou and Oreopoulou (2012) found that polar (paprika, marigold, bixin) and hydrophobic (*β*-carotene, lycopene) carotenoids exerted antioxidant effect measured by TBARs test during thermal accelerated autoxidation (60ºC) of sunflower oil-in-water emulsions stabilized by Tween 20.

Similarly to TBARs, the results of hexanal content after 4 weeks of storage showed that BHA and perilla extract (P_C2: 320 ppm) were the most effective antioxidant, followed by Trolox and P_C1. The protective effect of *P. frutescens* should be attributed to its content of the well recognised antioxidant like hydroxycinnamic acids and possibly other polyphenols. In the literature, other authors have described the antioxidant effect of caffeic acid and rosmarinic acid in model food emulsions. Caffeic acid (5 mmol/kg emulsion) showed good antioxidant properties in both 30% sunflower oil-in-water and 20% water-in-sunflower oil emulsions (pH 5.4) during storage at 50ºC (Conde et al., 2011). In the model corn oil-in-water emulsions rosmarinic acid (50µM) inhibited hexanal formation during storage at 55ºC for 24 days (Alamed et al., 2009).

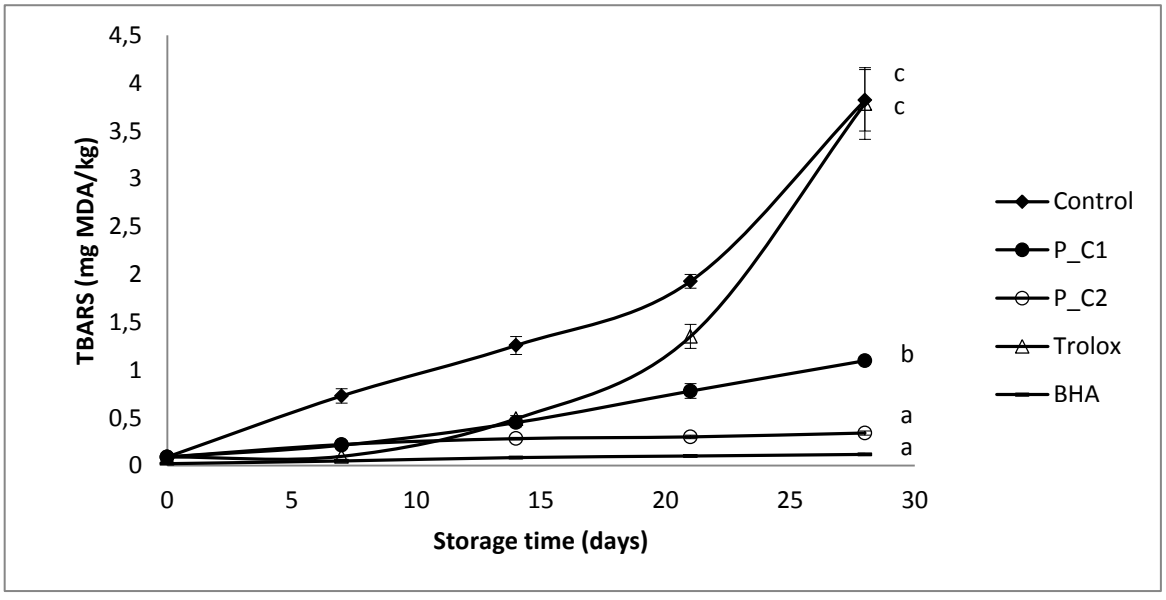

Figure 7.4 Evolution of secondary oxidation (TBARs) in model food system with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).
[a-c] Values with different letters at 30[th] day of storage are significantly different ($p<0.05$).

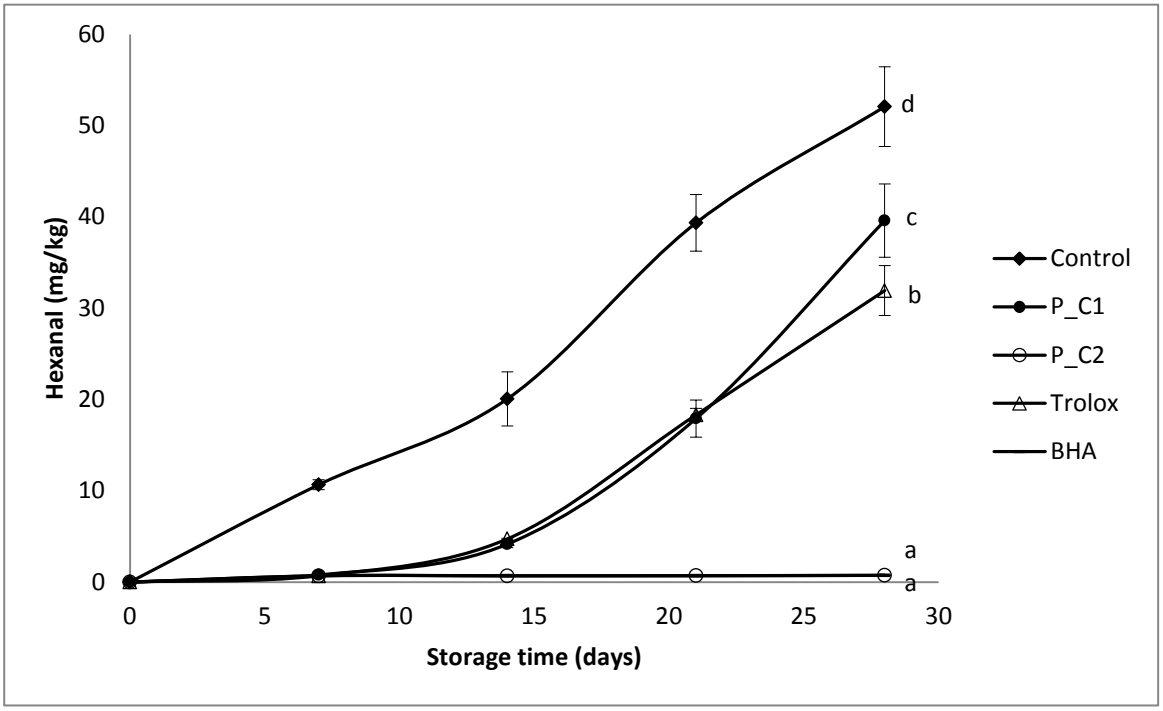

Figure 7.5 Evolution of secondary oxidation (hexanal content) in model food system with different concentration of perilla ethanolic extracts (C1: 80 ppm and C2: 320 ppm).
[a-d] Values with different letters at 30[th] day of storage are significantly different ($p<0.05$).

In the present study positive correlations between TBARs and hexanal (R^2 = 0.9106) levels and also between PV and both TBARs and hexanal (R^2 = 0.9446 and R^2 = 0.9431, respectively) levels in oil-in-water emulsions were found.

Different extracts of *P. frutescens* have demonstrated free-radical scavenging (Hong & Kim, 2010; Kang & Lee, 2011; Meng et al., 2009; Müller-Waldeck et al., 2010) and antioxidant action in rats (Feng et al., 2011; Yang et al., 2013). These properties of perilla have been associated with cinnamic acid derivatives and other polyphenolic compounds. Caffeic acid (CA) and rosmarinic acid (RA) are the major compounds in *P. frutescens* that showed the hepatoprotective effect against t-BHP-induced oxidative liver damage. *In vitro* and *in vivo* treatments with perilla leave extracts and with combined CA and RA gave almost the same efficacy of liver protection against oxidative stress. *In vivo* treatment of combined CA and RA resulted in a more than proportional increase in antioxidant enzymes and reduced levels of indicators of hepatic toxicity, compared to CA only treatment suggesting that the stronger hepatoprotective effect of perilla is brought about by the combination of CA and RA. In an *in vivo* study, samples pretreated with 1000 mg/kg of body weight (BW) of perilla extract showed no signs of toxicity. After the

conversion of the effective dosage in rats into a dose based on the surface area of humans they obtained the perilla extract equivalent in humans of 162 mg/kg BW, which equates to 9.7 g of perilla extract o 205 g of perilla fresh leaves (Yang et al., 2013).

The concentration of the perilla extract was selected so as not to exceed the non-toxic pharmacological doses. Therefore, the addition of 320 mg/kg of perilla extract to food emulsions would serve a twofold purpose: (i) to support the pharmacological doses of these extracts by using those food emulsion system as vectors; and (ii) to improve the fat stability and hence the nutritional properties of those emulsion systems.

7.4 Conclusions

This study showed that the extract of *Perilla frutescens* is a potential source of natural antioxidant to be used as a lipid oxidation inhibitor in food industry. In addition, food emulsions appear to be useful vectors in supplying the daily dosage of *P. frutescens* extract in consumers, which may positively affect their health. Further research into the enrichment of food products with bioactive substances extracted from *P. frutescens* should be conducted because there is still not sufficient knowledge about their activity during food processing, nor about their interactions with other food components.

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8 Antioxidant properties of *Artemisia annua* extracts in model food emulsions

8.1 Introduction

Lipid oxidation is of great concern to the consumer because it causes physical and chemical deterioration of food quality, such as undesirable changes in taste, texture, appearance and development of rancidity, losses of important nutritional values and formation of potentially harmful components including free radicals and reactive aldehydes (Alamed et al., 2009; Conde et al., 2011). Especially, this process is favored in oil-in-water emulsions because of the large contact surface between the oxidizable lipid hydroperoxides in emulsion droplets and water-soluble prooxidants resulting in the propagation of oxidation reactions (Waraho et al., 2012). To avoid this problem, synthetic antioxidants are commonly used, such as butylated hydroxytoluene and butylated hydroxyanisole (Brewer, 2011). However, in recent years there has been an increasing interest in the use of naturally occurring substances for the preservation of food. Aromatic plants have been the subject of study, particularly by the chemical, pharmaceutical and food industries, because of their potential use in food for two principal reasons: (i) safety considerations regarding the potentially harmful effects of the chronic consumption of synthetic compounds in food and beverages, and (ii) "natural" additives are perceived as beneficial for both quality and safety aspects and also possible beneficial effects on human health (Viuda-Martos et al., 2010).

Artemisia annua (*Asteraceae* family) commonly known as "annual wormwood" is a plant used for many centuries in Chinese folk medicine for the treatment of malaria and fever. Its health-promoting effects have been mainly attributed to its content of artemisinin, a sesquiterpene lactone used as the raw material for production of artemisinin-based combination therapy, used against drug-resistant *Plasmodium falciparum* in areas where malaria is endemic. *A. annua* is also a rich source of antioxidant flavonoids that are thought to play an important role in potentiating the effects of artemisnin drugs against cancer and parasitic diseases (Ferreira, Luthria, Sasaki, & Heyerick, 2010). Moreover, *A. annua* leaves have a high content of essential oil (EO) containing cineole, α-pinene, camphene, camphor and artemisia ketone (Radulović et al., 2013). The essential oil of *A. annua* is referenced as having antifungal and antimicrobial activity (Ćavar, Maksimović,

Vidic, & Parić, 2012). *A. annua* also shows anti-inflammatory, antipyretic (Huang et al., 1993), antioxidant (Kim et al., 2009), anticancer (Chan, Singh, & Lai, 2013; Singh, Ferreira, Park, & Lai, 2011) and cytotoxic (Nibret & Wink, 2010) activities. Although not yet reported in the literature, *A. annua* extracts, being a rich source of various phenolic compounds could therefore be incorporated in model emulsions as a source of natural antioxidant to prolong quality and stability.

The aim of this paper is to report a study of the antioxidant properties of *Artemisia annua* extracts in model emulsions stored for long periods (45 days), which can be representative of real food systems and their expected shelf life. Lipid oxidation was determined by following the formation of peroxide values (PV) as the primary oxidation products and thiobarbituric acid reactive substances (TBARs) as the secondary products.

8.2 Materials and Methods

8.2.1 Materials

Artemisia annua was grown in a greenhouse (Balaguer, Spain). Leaves of *A. annua* were collected, dried and ground to a homogenous powder in collaboration with the company Pàmies Hortícoles. Refined sunflower oil was purchased in a local market. All reagents and chemicals were of analytical grade supplied by Sigma–Aldrich Company Ltd. (Gillingham, UK) or Panreac (Barcelona, Spain).

8.2.2 Extraction

Air-dried and finely ground *Artemisia annua* was weighed (2 g) and extracted with 50 mL of ethanol-water mixture at 50:50 (v/v). The mixture was stirred continuously for 24 h at 4°C. After that, all samples were centrifuged (Sigma 6K10, Osterode am Harz, Germany). Part of the supernatant was used to determine the antiradical capacity. The volume of the remaining supernatant was measured and the solution was evaporated, frozen at -80°C for 24 hours and lyophilized for 3 days. Samples were then weighed and kept protected from light in a desiccator until used to prepare an oil-in-water emulsion system.

8.2.3 Total phenol and flavonoid content

Total polyphenol content (TPC) of extracts was determined by colorimetry following the Folin-Ciocalteu method (1999). The absorbance was measured at 725 nm using a UV–vis

spectrophotometer (Fluostrar Omega, Perkin-Elmer, Paris, France) and the results were expressed in gallic acid equivalents, GAE, using a gallic acid standard curve (10-70 µM).

Total flavonoid content (TFC) of extracts was measured according to the method of Zhishen et al. (1999). The absorbance at 510 nm was measured using spectrophotometer UV-4201/20 (Zuzi, AuxiLab, S.L., Navarra, Spain). Values were determined from a calibration curve prepared with catechin (ranging from 6 to 60 mg/L) and expressed as mg of catechin equivalent per gram of dry/fresh weight of plant (CE/g DW/FW).

8.2.4 Antioxidant capacity determination

Three different methods were used for the evaluation of the antioxidant activity of the extracts: 2,2'-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid ($ABTS^{\bullet+}$) assay (Almajano et al., 2008), Oxygen Radical Absorbance Capacity (ORAC) assay (Skowyra et al., 2014) and Ferric Reducing Antioxidant Power (FRAP) method (Gallego et al., 2013). Results were expressed as µmol of Trolox equivalent (TE) per gram of dry weight of plant (DW).

8.2.5 Liquid Chromatography – Mass Spectrometry

LC-MS analyses of the *A. annua* extracts were carried out using LC-QTOF-MS instrument, acquired from Agilent (Wilmington, DE, USA). The LC was an Agilent 1200 Series, consisting of a vacuum degasser unit, an autosampler, two isocratic high pressure mixing pumps and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source. The mobile phase was composed of 0.1 % formic acid (v/v) in water (eluent A) and 0.5% formic acid (v/v) in acetonitrile (eluent B). Separations were performed on a reversed-phase Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 3.5 µm) acquired from Agilent and connected to a C18 (4 mm × 2 mm) guard cartridge supplied by Phenomenex (Torrance, CA, USA). The temperature of the column was maintained at 30°C, the mobile phase flow was 0.2 ml/min, and the following gradient was used: 0-10 min, 3% B; 10-25 min, 100% B; 27-38 min, 3% B. The injection volume for samples was 10 µl. Nitrogen (99.999%), used as nebulising (35 psi) and drying gas (330°C, 10 l min^{-1}) in the dual ESI source, was provided by a high purity generator (ErreDue srl, Livorno, Italy). Nitrogen (99.9995%), for collision-induced dissociation experiments (MS/MS measurements), was purchased from Carburos Metálicos (A Coruña, Spain). The QTOF instrument was operated in the

2 GHz (Extended Dynamic Range, mass resolution from 4500, at m/z 100, to 11000, at m/z 900) mode and compounds were ionized in positive ESI, applying capillary and fragmentor voltages of 3500 and 160 V, respectively. A reference calibration solution (Agilent calibration solution A) was continuously sprayed in the source of the QTOF system, through a second nebulizer. The Mass Hunter Workstation software was used to control all the acquisition parameters of the LC-ESI-QTOF-MS system and also to process the obtained data. The compounds selected were followed operating the system in the MS mode. Thus, full scan MS spectra were acquired in the range from 100 to 1700 m/z units, during the whole chromatographic run, considering an acquisition rate of 1.4 spectra s^{-1}. After identifying the caffeic acid, the apigenin and the rutin, their full scan (70- 500 m/z) MS/MS spectra were acquired in further injections. To this end, their $[M + H]^+$ parent ions were fragmented using collision energies between 10 and 40 eV. Therefore, TPs identification was based on the accurate masses, isotopic abundances and spacing of signals in their ($[M + H]^+$) cluster of ions, obtained in the MS mode, as well as, on their MS/MS fragmentation patterns and the exact mass of products ions.

8.2.6 Oil-in-water emulsion system

8.2.6.1 Removal of tocopherols from sunflower oil

Tocopherols were removed from sunflower oil by column chromatography using activated alumina, as described by Yoshida et al. (1993). The oil was stored at -80 ºC prior to emulsion preparation (up to 2 days).

8.2.6.2 Preparation of emulsions and storage conditions

Oil-in-water emulsions were prepared with 1% of Tween 20 as emulsifier and 10% of sunflower oil. Emulsions were prepared by dropwise addition of oil to the water phase, with sonication using a UP200S ultrasonic (Hielscher Ultrasonics GmbH, Teltow, Germany) while cooling in an ice bath for 10 min. It was necessary to repeat sonication 7 times (7 x 10 minutes) to have enough volume of emulsion. Freeze-dried powder of the *A. annua* extract was redissolved in ethanol 50% (v/v) and added directly to the emulsion and homogenized, obtaining final concentrations of 0.20, 0.65 and 2 g/L (C1, C2 and C3, respectively). For the negative control no extract was added, and the positive controls were prepared with Trolox (0.02 g/L) and BHA (0.02 g/L) dissolved in ethanol.

All emulsions were stored in triplicate in 30 mL amber bottles in the dark, with constant elliptical movement and allowed to oxidize at 32 ± 1 ºC for 45 days.

8.2.6.3 Measurement of primary oxidation by peroxide value (PV) and pH

Peroxide value (PV) was measured periodically (every 2 or 3 days during the time of storage) using aliquots of 0.007–0.01 g of each sample and determined by the ferric thiocyanate method (Frankel, 1998), after calibrating the procedure with a series of oxidized oil samples analyzed by the AOCS Official Method Cd 8-53 (1997).

The pH of the samples was measured (pH-meter GLP21, Criston Instruments, Barcelona, Spain) as a parameter to investigate its correlation with PV.

8.2.6.4 Measurement of secondary oxidation by TBARs method

The thiobarbituric acid reactive substances (TBARs) assay was performed as described by Maqsood and Benjakul (2010) with some modifications. One mililiter of oil-in-water emulsion sample was mixed with a TBARs solution containing 0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl solution (5 mL). The samples were placed immediately in an ultrasonic bath (Prolabo brand equipment) for 5 min and then heated in a water bath (95ºC) for 10 min. The mixture was centrifuged (Sigma 3K30, Sigma Laborzentifugen GmbH, Osterode am Harz, Germany) at room temperature at 4000 rpm for 10 min. The absorbance of the supernatants was measured at 532 nm (Spectrophotomter UV-4201/20, Zuzi, Navarra, Spain). The TBARs values were expressed as mg of malondialdehyde (MDA) per kg of emulsion calculated using 1,1,3,3-tetraethoxypropane (Sigma-Aldrich, St. Louis, Missouri, USA) as the standard.

8.2.7 Statistical analysis

TPC, TFC, $ABTS^{+}$, ORAC and FRAP measurements were performed in triplicate on triplicate samples. PV and TBARs measurements were performed once on triplicate samples.

Mean values for different parameters were calculated and compared by analysis of variance (one-way ANOVA) using commercial software (Minitab 16). Moreover, statistical differences between mean values were identified at the 95% of confidence level ($p<0.05$). Person's correlation analysis was performed using the same statistical package.

8.3 Results and Discussion

8.3.1 Phenolic content and in-vitro antioxidant activity of extract

The total polyphenols (TPC) and flavonoids (TFC) in extracts of *A. annua* leaves obtained with 50% ethanol are shown in Table 8.1. The *A. annua* extract contained 23.36 ± 0.92 mg gallic acid (GAE)/g dry weight (DW) and 2.68 ± 0.07 mg catechin/g DW (TPC and TFC, respectively).

A recent paper on the analysis of extracts of *A. annua* (Gouveia & Castilho, 2013) found a TPC values (384.1 ± 6.7 to 521.2 ± 5.4 mg GAE/100 g DW) for methanol and acetone extraction, respectively, much lower than what we report here for ethanolic extract. However, studies involving hexane and methanol extraction of *A. annua* leaves have reported higher values than those obtained in the present study, in the range of 90.12-134.50 mg GAE/g DW (Iqbal et al., 2012) . In addition the same authors found higher TFC value (6.14 mg epicatechin/g DW) in the methanolic extract. Consequently, the extraction method and the solvent used play a key role in the extraction of polyphenols and flavonoids from plant material.

Table 8.1 Polyphenol and flavonoid content and antioxidant activity of *A. annua* extracts.

Method	Dry weight	Fresh weight
Total polyphenol content (mg GAE/g)	23.36 ± 0.92	39.71 ± 1.56
Total flavonoid content (mg CE/g)	2.68 ± 0.07	4.55 ± 0.12
ABTS (µmol TE/g)	314.99 ± 7.70	535.48 ± 13.09
ORAC (µmol TE/g)	736.26 ± 17.55	1251.64 ± 29.83
FRAP (µmol TE/g)	212.18 ± 6.02	360.70 ± 10.23

*Results are expressed as mean ± standard deviation (n=3).

Antioxidant activity of the extracts from *A. annua* was assessed by three different methods: ABTS, ORAC and FRAP. The use of several methods provides more comprehensive information about the antioxidant properties of the original product because there are substantial differences in sample preparation, extraction of antioxidants (solvent, temperature, etc.), selection of end-points and expression of results (Viuda-Martos et al., 2010). For the ABTS assay the value obtained was 314.99 ± 7.70 µmol TE/g DW, a value 2 times lower than that found in the ORAC assay which was 736.26 ± 17.55 µmol TE/g DW. It is quite usual to obtain higher values in the ORAC test, due to differences in the sensitivity of these methods. Finally, for the FRAP assay the value

found was 212.18 ± 6.02 µmol TE/g DW. Gouveia and Castillo (2013) found the ABTS value of 477.0-2197.3 µM TE/100 g DW in *A. annua* leaves using extraction with methanol and acetone, respectively. Also Zheng and Wang (2001) found the ORAC value (15.69 ± 0.57 µM TE/g fresh weight) in the phosphate buffer extract much lower than what we report here for the alcoholic extract. Viuda-Martos (2010) described the ferric reducing capacity and metal chelating ability of the *A. annua*, finding strong reducing power and effectivity in metal chelating (62.25-98.03%) of essential oils from *A. annua*. They also reported determination of oxidative stability of fat (Rancimat assay), finding that 5-50 g/L *A. annua* essencial oils showed pro-oxidant activity.

A few recent reports indicated that *A. annua* was one of the four medicinal plants with the highest ORAC level, the ORAC value of *A. annua* leaves and inflorescences extracts was reported as 1,125 and 1,234 µM TE/g, respectively, which is half to two thirds of the ORAC of oregano extracts (Ferreira et al., 2010).

LC-MS analysis of the plant extract of *A. annua* showed the presence of several phenolic compounds quantified in the following increasing order: caffeic acid, rutin and apigenin (Table 8.2). The concentrations of caffeic acid (1.352 µg/g DW), rutin (0.765 µg/g DW) and apigenin (0.135 µg/g DW) in *A.annua* extract were lower than those reported in the literature. Carvalho et al. (2011) reported that the *A. annua* leaves conteined 80 µg/g of DW of catechins, 2 µg/g of DW of flavonols, 75 µg/g of DW of hydroxycinnamic acids and 430 µg/g of DW of hydroxybenzoic acids. Carbonara et al. (2012) found in water extracts of *A. annua* 3.11 ± 0.02 – 4.10 ± 0.06 mg/g DW of caffeic acid. Morover, Ivanescu et al. (2010) reported that *A. annua* had 1.144 mg/100 g DW of apigenin.

Table 8.2 LC-MS parameters and amount of selected antioxidant compounds in *A. annua* extracts.

Compounds	Rt (min)	Linear regression equation	R^2	Linear range (ppm)	MS (m/z) [M-H]	Content µg/g DW
Rutin	5,33	y= 333.54x + 2184.6	0.998	0.1-1	609	0.764
Caffeic acid	5,41	y= 588.03x + 198.38	0.999	0.1-1.5	179	1.353
Apigenin	7,85	y= 1028.4x + 37085	0.991	0.1-0.5	269	0.135

8.3.2 Antioxidant activity of extracts in model emulsion system

In this study, to accelerate the oxidative damage, emulsions were stored at 32 ± 1°C. Oxidative stability was assessed by periodic analysis of primary and secondary oxidation products (measured by the peroxide and the thiobarbituric acid reactive substances values, respectively). In addition the change in pH was monitored, since pH tends to fall during oxidation.

Peroxide values in the oil emulsions increased significantly faster in the sample without any antioxidant addition (Fig. 8.1), reaching 10 meq hydroperoxides/kg of emulsion (this value is the allowed limit for products containing edible fats) after four days. The next samples to reach this level of deterioration were Trolox at 0.02 g/L (after 10 days), Art_C1 at 0.20 g/L (after 16 days) and Art_C2 at 0.65 g/L (after 28 days). Other samples: Art_C3 and BHA were stable until the end of the experiment (after 45 days, PV was < 10 meq/kg). *A. annua* extracts added to oil-in-water emulsions were very effective in stablilzing the emulsion with 2 g/L *A. annua* extract being similar to BHA (0.02 g/L) in activity during 45 days of storage at 32°C.

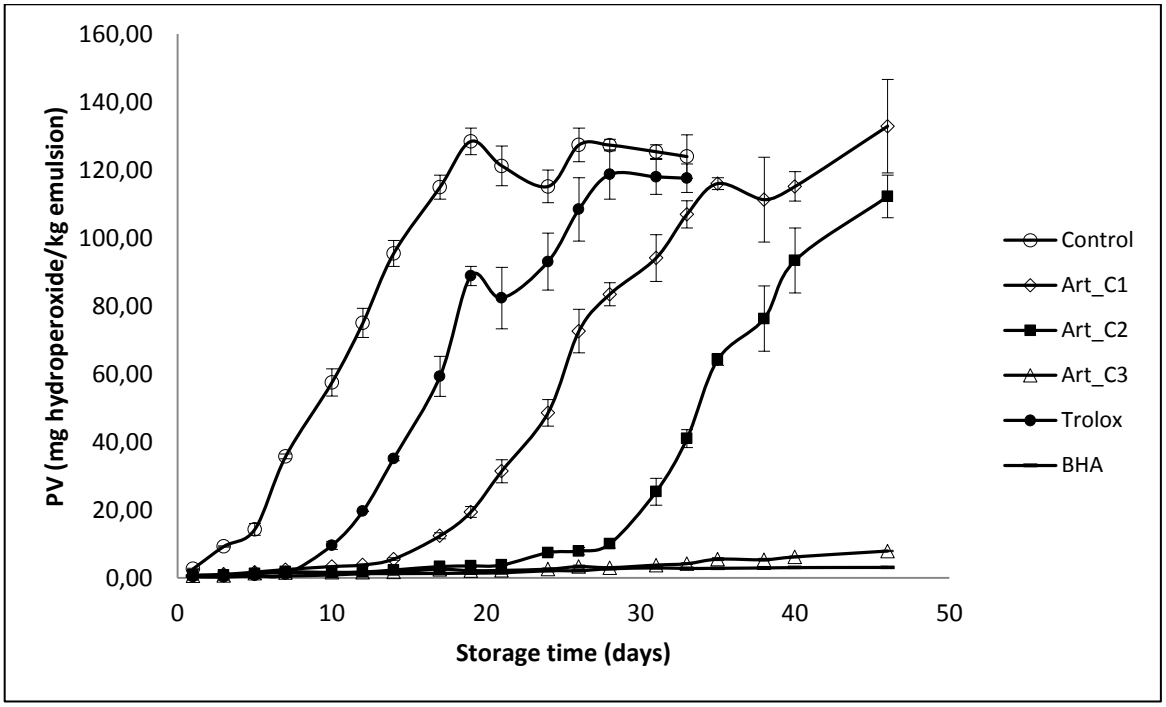

Figure 8.1 Evaluation of primary oxidation (peroxide value) in a model food system with different concentrations of *A. annua* (C1: 0.20 g/L; C2: 0.65 g/L and C3: 2 g/L).

Kiokias et al. (2009) reported peroxide values between 45.60 and 51.15 meq/kg after two months in 10% sunflower oil-in-water emulsions with 2 g/L of different carotenoids including *β*-carotene, lycopene, paprika, lutein and bixin. Ramful et al. (2011) found that *Eugenia pollicina* leaf extract at a concentration of 0.02% was also effective in slowing down hydroperoxide formation in soybean oil emulsion during 13 days of storage at 40ºC. Roedig-Penman et al. (1997) reported that tea extracts added to sunflower oil-in-water emulsion were very effective in its stabilization, the tea extract (0.03%) being similar to BHT (0.02%) and taking 40 days of storage at 30°C to reach a PV of 30 meq/kg.

pH can affect oxidative reactions by influencing prooxidant (e.g., iron solubility increases with decreasing pH) and antioxidant (the pH can alter the charge of antioxidants, which can affect solubility and chelation capacity) activity. The pH of oxidation models should therefore be similar to the food of interest (Decker, Warner, Richards, & Shahidi, 2005). In addition, since it is known that many antioxidant molecules are less effective when the pH is low (Sun et al., 2011), this parameter was also measured as a potential indicator of oil-in-water emulsions oxidation. From an initial average value of 5.5, the samples without any antioxidant addition and with Trolox tended to stabilize their pH at 2.60 and 2.74, respectively, after 45 days (Fig. 8.2). In the Art_C1, Art_C2, Art_C3 and BHA samples the pH slowly decreased during storage, but in Art_C1 and Art_C2 it decreased rapidly after 25 and 33 days, reaching the value of 2.90 and 3.24, respectively. Observing this relationship confirmed that the pH fell as PV increased. Gallego et al. (2013) and Sorensen et al. (2008) reported that lipid oxidation increased when pH was decreased from 6 to 3 in a 10% oil-in-water emulsion.

Secondary oxidation products in the emulsions were monitored by measurement of the TBARs (Fig. 8.3). After 6 weeks, TBARs values in emulsions containing *A. annua* extracts and BHA were lower than that those in the control (4.27 mg MDA/kg) and the Trolox-containing sample (3.80 mg MDA/kg). BHA was the most effective antioxidant followed by *A. annua* extract Art_C3, Art_C2 and Art_C1.

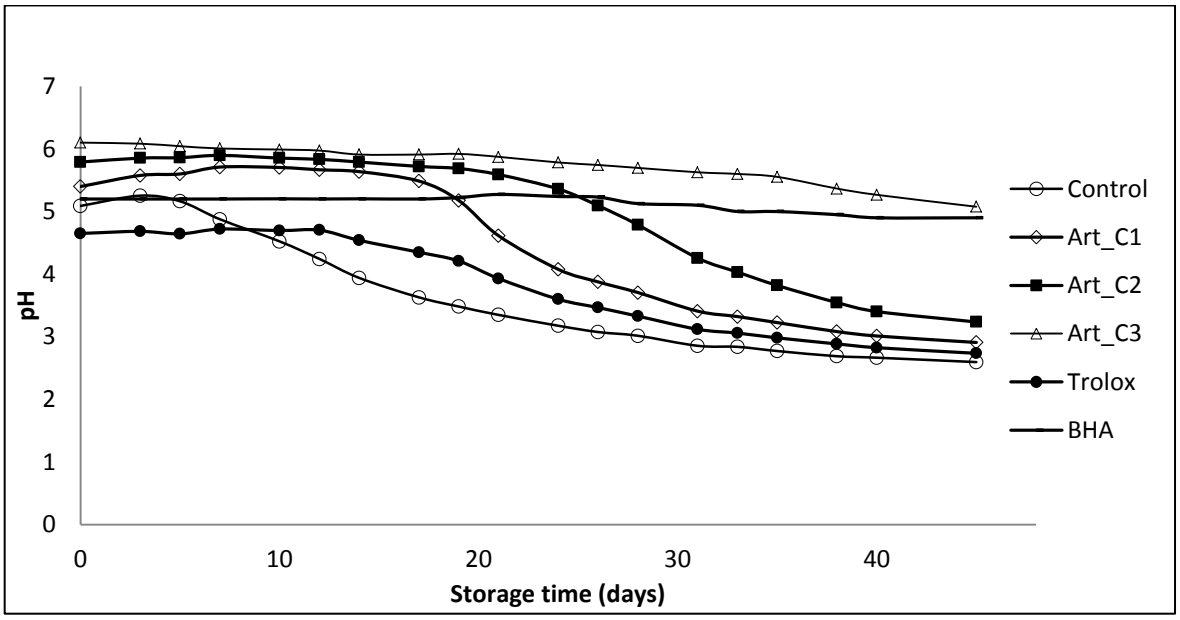

Figure 8.2 Evaluation of pH in a model food system with different concentrations of *A. annua* (C1: 0.20 g/L; C2: 0.65 g/L and C3: 2 g/L).

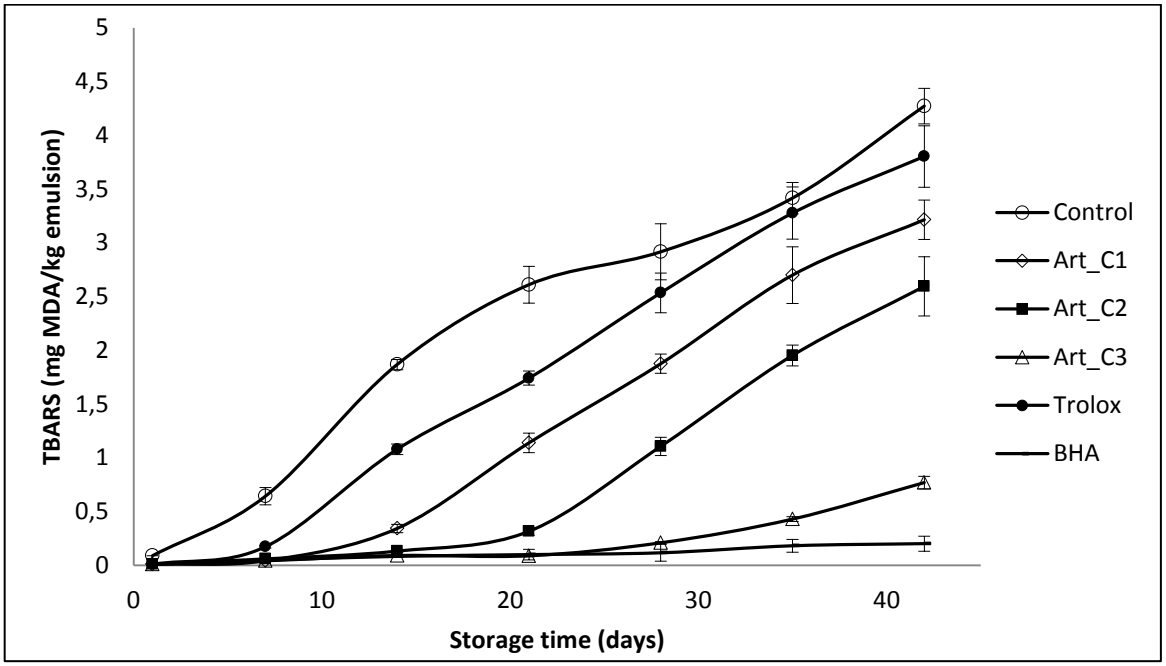

Figure 8.3 Evaluation of secondary oxidation (TBARs) in a model food system with different concentration of *A. annua* (C1: 0.20 g/L; C2: 0.65 g/L and C3: 2 g/L).

Garcia-Iñiguez et al. (2010) reported that a lyophilized aqueous extract of *Melissa officinalis* (lemon balm) at 620.6 ppm was as efficient as BHA at 200 ppm in controlling the TBARs formation in oil-in-water emulsions made with a mixture of algae and lineseed oils upon storage during 15 days at 20ºC. Dimakou and Oreopoulou (2012) found that polar (paprika, marigold, bixin) and hydrophobic (*β*-carotene, lycopene) carotenoids exerted antioxidant effect measured by TBARs test during thermally

accelerated autooxidation (60ºC) of sunflower oil-in-water emulsions stabilized by Tween 20.

In the present study positive correlation between PV and TBARs (R^2 = 0.9200) levels in oil-in-water emulsions was found.

The activity of phenolic compounds as antioxidants in food systems (such as oil-in-water emulsions) depends not only on the structure (i.e., number and position of hydroxyl groups bound to the aromatic ring) and chemical reactivity of the phenolics but also on other factors such as their physical location, interactions with other food components, and environmental conditions, for example pH (Conde, Gordon, et al., 2011; Medina et al., 2012; Sørensen et al., 2008). Natural plant antioxidants can protect food components from oxidation under the stress of heating and storage. The most effective antioxidants are those that interrupt the free radical chain reaction. Usually containing aromatic or phenolic rings, these antioxidants donate $H^{\bullet}$ to the free radicals formed during oxidation becoming radicals themselves. These radical intermediates are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinone structures. In addition, in many of the phenolics positions suitable for molecular oxygen attack are not availabe. Both synthetic (BHA and BHT) and natural plant antioxidants contain phenolic (flavonoid) functions. Plant extracts with antioxidant activity generally quench free radical oxygen with phenolic compounds as well (Brewer, 2011). However, the addition of polyphenols to lipid dispersions has been shown to result not only in antioxidant effects (Almajano et al., 2007), but also in pro-oxidant activity (Zhou & Elias, 2013).

Phenolic compounds such as caffeic acid, rutin and apigenin have received increasing interest due to their potential antioxidant activity. Caffeic acid has a single aromatic ring with two –OH groups that are capable of donating $H^{\bullet}$. In addition it is a polar compound with a strong ability for chelating metals (Brewer, 2011). Rutin is a compound that contains an *o*-diphenol group in their molecular structure (*o*-diphenol groups are able to chelate metal ions such as iron) (Sørensen et al., 2008).

The antioxidant capacity of natural extracts in food emulsions bas been ascribed to a number of influential factors, including the different polarities and antiradical activities of mixed phenolics. The presence of water in the emulsion results in the partition of antioxidants between polar and apolar phases, a fact influencing the antioxidant activity.

According to the "polar paradox", hydrophilic antioxidants are more effective in nonpolar media, whereas lipophilic compounds are better antioxidants in polar media. However, several authors have reported that some compounds do not comply with the polar paradox and interpreted the behavior of phenolic compounds in emulsified systems using a different approach known as the "cutoff theory" (Conde et al., 2011; Poyato et al., 2013). Sorensen et al. (2008) reported that caffeic acid and rutin inhibited the development of PV during the entire storage period in Citrem-stabilized emulsions at pH 6. Furthermore, the most water-soluble compound, caffeic acid, showed different effects depending on pH and emulsifier type. Thus, it was a strong pro-oxidant at pH 3 (with or without iron), but at pH 6 its effect depended on the emulsifier type and on the presence of iron. In addition Medina et al. (2012) reported that at pH 6, caffeic acid was able to reduce the amount of peroxides formed in emulsions containing Tween, but increased the formation of volatiles. Conde et al. (2011) found that caffeic acid (5 mmol/kg emulsion) showed good antioxidant properties in 30% sunfloweroil-in-water emulsions at pH 5.4 during storage at 50 °C. The same author (Conde et al., 2011) reported that higher concentrations of rutin and apigenin in the rafined extracts produced from chestnut burs retarded the formation of hydroperoxides in oil-in-water emulsions.

The ability of a compound to inhibit lipid oxidation could be influenced by its interactions with other antioxidants (Alamed et al., 2009). Synergy between antioxidants has been reported in a range of different media, including oils, emulsions, liposomes, microemulsions, fish and meat muscles. In some reports, the effects of antioxidants used in a combination could only be described as additive, but the term synergy should be restricted to situations where the mixture of antioxidants has a greater impact than the sum of their separate effects. Synergy between antioxidants may vary both with the medium and the nature of the lipids. Caffeic acid was effective in protecting α-tocopherol in retarding lipid oxidation in the fish muscle (Gordon, 2010; Iglesias et al., 2009). α-Tocopherol showed a strong synergistic effect with queretin in the methyl oleate in water emulsion, but the effect was reduced in phospolipd liposomes and the combination of α-tocopherol and quercetin had a shorter induction time than quercetin alone, when the oxidative stability was assessed in oil by the Rancimat test (Becker, Ntouma, & Skibsted, 2007).

8.4 Conclusions

This study showed that the extract of *Artemisia annua* provides protection against the oxidative deterioration of oil-in-water emulsion. In addition, food emulsions appear to be useful vectors in supplying the daily dosage of *A. annua* extract in consumers, which may positively affect their health. Moreover, considering consumer's preference for antioxidants from natural sources, these results could offer the basis for their more systematic use by food industry. Further research into the enrichment of food products with bioactive substances extracted from *A. annua* should be conducted because we still have no sufficient knowledge about their activity during food processing, no about their interactions with other food components.

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9 Characterization of phytochemicals in petals of different colours from *Viola wittrockiana* Gams.

9.1 Introduction

The traditional use of flowers in food has been thoroughly documented. Several ancient world cultures used many species for medical uses and often incorporated them as part of their diet. In Spain the use of flowers in food was not very common until the end of the last century, when they had been commonly incorporated into dishes, mainly as ornamental elements. This popularity has encouraged their use as additives in food industry due to their well-known potential health benefits, in order to obtain new and attractive functional products (Sanchez-Mata et al., 2012). At that time studies were performed in order to analyze the consumers' acceptability of flower petals as edible vegetables. Parameters influencing the consumers were analyzed and petal colour obtained the highest influence rate (between 63 and 95 %) (Kelley, Behe, Biernbaum, & Poff, 2001).

Heartsease (*Viola tricolor* L.) is a well-known medicinal plant. Its biological activities are supposed to be related to its antioxidant capacity. Garden pansies (*Viola wittrockiana* Gams) are common and long cultivated ornamental hybrid plants developed in the 19th century by crossbreeding *Viola tricolor L.* in order to produce more attractive flowers. The plants are known to produce petals of many possible colours. Vukics et al. (2008a) found that both, wild and garden pansies (*V. tricolor* and *V. wittrockiana)* contained similar amounts of antioxidant substances. Attractive flowers and antioxidant capacity make garden pansy a suitable candidate to be used as functional food product (Vukics, Kery, & Guttman, 2008).

Violae are sources rich in phytochemical compounds which play important roles in plant metabolism, growth and reproduction, while some of them bring about protection against pathogens, predators and UV radiation. Some of these compounds, such as polyphenols, were shown to cause important nutritional and health benefits in humans (Kaisoon, Siriamornpun, Weerapreeyakul, & Meeso, 2011). From a nutritional standpoint, polyphenols exhibit a wide range of physiological properties, such as antiallergenic, antiatherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects. They have also been linked to oxidative stress

disease prevention in Alzheimer, Parkinson, atherosclerosis and various cancers (Feng & Wang, 2012; Schiffrin, 2010). Studies have demonstrated that dietary polyphenols can act as scavengers of free radicals, inducing, inhibiting or modulating the signal of transduction of antioxidant enzymes or chelating metal ions (Han, Shen, & Lou, 2007). For a complete determination of phenolic content and antioxidant activity, quantitative and qualitative information is needed. Several studies analyzed *Viola tricolor* L. (*Violaceae*) extracts by TLC or HPLC combined with UV detection but results were not specific enough for the identification of flavonoid glycosides. For this reason Papp et al. (2004) examined ethanolic petal extracts by HPLC-DAD and on-line mass spectrometry (ESI-MS or APCI-MS) and five peaks were identified: quercitin, isoquercitin, hyperoside, rutin, and luteolin-7-*O*-β-glucoside (Papp et al., 2004). Vukics et al. (2008 c) performed extraction of heartsease herb with chloroform (fraction A) and then with ethanol (fraction B) in an ultrasonic bath. According to their results, the two major pansy flavonoids were violanthin and rutin. In addition, the same authors stated that flowers of heartsease are a potential source of natural antioxidants and that this tissue showed higher antioxidant activity than the herb and leaves. Moreover, a significant correlation between the flavonoid content and antioxidant activity was found (Vukics et al., 2008 b). The same authors made a comparative study of antioxidant capacity of garden pansies of three different flower colours and wild pansy (Vukics et al., 2008a). Previous papers had focused on the study of the antioxidant activity of herb and petals of wild and garden pansies but did not identify most of their flavonoids.

The aim of this research was to investigate more deeply the composition of polyphenols, flavonoids and anthocyanins in flowers of *Viola wittrockiana* and to establish relationships between the colour, the methodology of extraction, polyphenols, flavonoids, total anthocyanins and antiradical activity *in vitro*. Flavonoids and anthocyanins were investigated using HPLC coupled with diode-array detection (DAD) and mass spectrometry (ESI-MS). The differences between three petal colours (yellow, red and violet) using three extraction solvents (water, 50% ethanol and 50% ethanol in acid media) are reported.

9.2 Material and methods

9.2.1 Chemicals and reagents

All the solvents employed, analytical and HPLC grades were purchased from Panreac (Barcelona, Spain). Standards and reagents such as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), gallic acid (GA) and rutin were purchased from Sigma-Aldrich Company, Ltd. (Gillingham, UK). Vainillin was obtained from Merck (Darmstadt, Germany). Standards for HPLC-DAD and HPLC-MS analysis were purchased from Extrasynthese (Genay Cedex, France). All other chemicals were purchased from common sources.

9.2.2 Plant material and preparation of extracts

Three petal colours of garden pansies (*Viola wittrockiana*), belonging to violet family *(Violaceae)*, were analysed: yellow, red and violet. Petals were grown in a greenhouse (Balaguer, Spain). Twelve different tissues of each variant of petal colours were manually collected, dried, and ground to a homogenous powder in January 2012 in collaboration with the company Pàmies Hortícoles, S.L., comprising three replicates. The powder was stored protected from light and moisture until the extraction.

Each variant of petal colour was treated with three types of solvent: water, 50% ethanol and 50% ethanol in acid media (hydrochloric acid was added to pH=1.5-2). The extracts were prepared according to the published procedure (Lopez et al., 2008). Futhermore, three different extractions were carried out for each petal colour in order to obtain representative quantitative results. For each extraction, 1.5 g of air-dried and homogenous powder of petals was macerated with 50 mL of each solvent. The mixture was stirred continuously for 24 h at 4 °C. After that, all samples were centrifuged (Sigma 6K10, Osterode am Harz, Germany). Part of the supernatant was introduced in a rotary evaporator until the complete evaporation of the organic solvent, frozen at -80 ºC for 24 h and lyophilized for 3 days to provide the sample to be used in the assays. Samples were then weighed and kept protected from light in a dessicator until use. There were no differences in extraction yield among the different extracts. Values ranged between 15 and 20% for all extracts.

9.2.3 Qualitative determination on DPPH free radical scavenging capacity by TLC

100 µg of crude extracts were spotted on silica-gel 60 F_{254} plates (Merck, Germany) and were developed in ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26). Three plates were prepared under same conditions, one for the antiradical test and the others to establish a relation between the antioxidant activity and the nature of the active compounds (Wagner S., 1996).

The first plate was sprayed with a methanolic solution of DPPH (2 mg/mL). The second plate was sprayed with vanillin–sulphuric acid (observed at visible) as a general reagent and the third plate was sprayed with natural products–PEG (view under 366 nm) to detect flavonoids.

9.2.4 Quantitative determination of antioxidant activity

Four different methods were used for the evaluation of the antioxidant activity of the extracts: DPPH radical-scavenging assay (Lopez et al., 2008), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) assay (Almajano, Carbo, Jimenez, & Gordon, 2008), Oxygen Radical Absorbance Capacity (ORAC) assay (Almajano, Delgado, & Gordon, 2007) and Ferric Reducing Antioxidant Power (FRAP) method (Skowyra et al., 2014). Results were expressed as mg Trolox (TE)/g of freeze-dried weight (FDW) extract. All measurements were performed with an automated microplate reader and 96-well plates using a spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France). Absorbance measurements were made in triplicate for each diluted solution.

9.2.4.1 DPPH assay

The DPPH assay determined the ability of extracts to scavenge the DPPH radical. A solution of DPPH (5.07 mM) in pure methanol was prepared. Appropriate dilutions were made for the study of the samples to allow the fall in DPPH concentration to be in the range 10-90 %. Then the solution of DPPH and samples (at a concentration of 10% v/v of sample and 90% of the radical) were added to the well of a microplate. Absorbance was measured at 517 nm, every 15 min for 75 min.

9.2.4.2 ABTS assay

The assay is based on the ability of an antioxidant compound to quench the $ABTS^+$ relative to that of a reference antioxidant such as Trolox. A stock solution of $ABTS^+$ radical cation was prepared by mixing ABTS solution with a potassium persulfate solution at 7 mM and 2.45 mM final concentration, respectively. The mixture was maintained in the dark at room temperature for 16 h before use. The working $ABTS^+$ solution was produced by dilution in 10 mM PBS (pH 7.4) incubated at 30 °C of the stock solution to achieve an absorbance value of 0.7 (±0.02) at 734 nm. An aliquot of 20 µL of diluted extract was added to $ABTS^+$ working solution (180 µL). For the blank and standard curve, 20µL of PBS or Trolox solution were used, respectively. Absorbance was measured by means of a UV–vis spectrophotometer (Fluostar Omega, Perkin-Elmer, Paris, France) at 734 nm immediately after addition and rapid mixing (t_0) and then every minute for 15 min. Readings at t = 0 min (t_0) and t = 5 min (t_5) of reaction were used to calculate the inhibition percentage value for each extract: % inhibition of the sample = $[(t_0 - t_5)/t_0] \times 100$ – [% inihibition of the blank]. A standard reference curve was built by plotting % inhibition value against Trolox concentration (2–32µM).

9.2.4.3 ORAC assay

Diluted extract (40 µL) was transferred by pipette into each well and then 120 µL of 1.34 µM fluorescein working solution in phosphate buffer (13.3 mM) at 37 °C were added to each sample. The plate was placed in a spectrophotometer and incubated at 37 °C. The initial fluorescence was recorded at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. 2,2′-Azobis(2-metylpropionamide) dihydrochloride (AAPH, 40 µL, 30 mM) was then added to each sample well and the fluorescence was measured immediately and every 2 min thereafter for 120 min. For the calibration curve, solutions of Trolox were prepared in the range of 8–58 µM. The ORAC value for each extract was calculated using a regression equation relating Trolox concentration and the net area under the fluorescence decay curve.

9.2.4.4 FRAP assay

The FRAP reagent was prepared with acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl, 40 mM) and $FeCl_3$ (20 mM). The proportions were 10:1:1 (v:v:v), respectively. A suitable dilution of the extract was added to the FRAP reagent (1:30, v:v) and incubated

at 37 °C. The assay was performed by means of an automated microplate reader (Fluostar Omega, Perkin-Elmer, Paris, France) with 96-well plates. The absorbance at 593 nm at time zero and after 4 min was recorded. The analysis was performed in triplicate and values were determined from a calibration curve of Trolox (ranging from 2.5 to 33 µM).

9.2.5 Total phenolic (TPC), total flavonoid (TFC) and total anthocyanin (TAC) content

To determine the TPC an originally developed method and improved by Prior et al. was used (Prior, Wu, & Schaich, 2005). Finally, the optical density of the blue-coloured resulting solution was measured at 765 nm using a Hewlett-Packard 8452A diode array spectrophotometer. Gallic acid calibration curve (2-14 mg/L) was used to determine TPC concentrations (y=0.076x-0.0175, r^2=0.9989). Results are expressed as mg of gallic acid equivalents (GAE)/g of freeze-dried weight.

TFC was determined using the method from German Pharmacopoeia. A known volume of sample reacted with $AlCl_3$ 20 mg/mL in 5% acetic acid in ethanol in 3:1 ratio. After 30 minutes absorbance was measured at 405 nm. The calibration curve was prepared with rutin (15-90 mg/L) and results are expressed as mg of rutin equivalents (RE)/g of freeze-dried weight (y=0.008x+0.0246, r^2=0.9971).

The quantification of TAC is based on the spectrophotometric measurement of absorbance at a specific wavelength, 520 nm. Malvidin 3-*O*-glucoside calibration curve (40-300 mg/L) was use to obtain final concentrations and data were expressed as mg of malvidin glucoside equivalents (ME)/g of freeze-dried weight (y=0.0031x–0.1511, r^2=0.9676).

9.2.6 HPLC analysis

Identification of all constituents was performed by HPLC-DAD and MS analysis by comparing the retention time, the UV and MS spectra of the peaks in the extracts.

9.2.6.1 HPLC-DAD analysis

HPLC analysis was performed on an Agilent 1200 chromatograph (Agilent Technologies, Germany). Compounds were separated on a C18 reversed-phase column (*4.6×250 mm, 5 µm,* Phenomenex, Torrance, CA, USA) and detected with a diode array detector (DAD). Injection volume was 10 µL and flow rate was 1 mL/min. Gradient elution was applied

with 0.1% formic acid-acidified water (A) and 0.1% formic acid-acidified acetonitrile (B) as solvents. Gradient conditions were as follows: 0 min, 90% A; 4 min, 85% A; 20 min, 80% A; 22 min, 10% A; 24 min, 90% A; 29 min, 90% A. The UV–vis spectra were recorded between 220 and 550 nm.

9.2.6.2 HPLC-MS analysis

The HPLC system described above was interfaced with a HP1100 MSD API-electrospray detector (Agilent Technologies, Palo Alto, CA, USA). The column and the chromatographic condition were the same as those used for the HPLC analysis. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: negative and positive ionisation mode, scan spectra from m/z 100 to 1000, was used with a gas temperature of 350°C, nitrogen flow rate of 10 L/min, nebulizer pressure 30 psi, quadrupole temperature 30°C and capillary voltage 3.5 kV. The collision energy was 60 eV.

9.2.6.3 Validation of HPLC method and quantitative determination of individual constituents

The HPLC method was validated in terms of linearity, limit of detection (LOD), limit of quantitation (LOQ) precision, specificity, and accuracy according to the International Conference on Harmonization (ICH) guidelines (Shabir, 2003).

The quantitative determination of flavonoids and anthocyanins was performed using the external standard method and these compounds were detected at 350 and 525 nm. The calibration curves were obtained with five samples of various concentrations by linear regression analysis. The equation of linear regression for flavonol derivatives was y=13.28x+3.20 (r^2=0.997) and for anthocyanin derivatives was y=10.33x+2.53 (r^2=0.998), using rutin and malvidin-3-*O*-glucoside as standards, respectively. Both detection limits were 0.1 μg/mL. The repeatability and reproducibility of the chromatographic separations was verified with several columns, and all chromatographic analyses were reproducible on a second HPLC. Extracts were analysed three times and each extract obtained was injected three times into the HPLC. The amount of flavonoids and anthocyanins in each extract was expressed in mg per gram of dried petals.

9.2.7 Statistical analysis

Results were expressed as mean ± standard deviation. All data were analyzed by the computer application SPSS for Windows (version 15.0, SPSS Inc., Chicago, USA). Statistical differences were evaluated with one-way analysis of variance (ANOVA) and Dunnett *post hoc* test. Differences at the level $P<0.05$ were considered to be significant. Correlations between total phenol contents, total flavonoid contents, total anthocyanin contents and antioxidant activity were established by regression analysis.

9.3 Results and discussion

Thin-layer chromatography (TLC) chemical screening is a simple, fast, and low-cost method of identification of chemical ingredients in plant extracts. In order to detect antioxidant activity in water, 50% ethanol and 50% ethanol in acid media extracts, a method based on the reduction of DPPH radical by TLC was carried out. The use of DPPH provides an easy and rapid way to evaluate antioxidant activity. DPPH is a free radical stable at room temperature, which produces a violet solution in methanol. In the DPPH free radical scavenging capacity assay by TLC, the extracts that produced yellow spots on the plates were considered to contain antioxidant agents. Some plants are rich in secondary metabolites, such as flavonoids, anthocyanins, phenolic acids and tannins. In all the extracts assayed high antioxidant/antiradical capacities were detected. After studying the plates sprayed with vanillin/H_2SO_4 and Natural Products reagents PEG it was deduced that the activity might be due mainly to phenolic compounds, flavonoids and anthocyanins.

A number of assays have been described in the literature for the measurement of total antioxidant activity of food, medicinal extracts and pure compounds (Dudonne, Vitrac, Coutiere, Woillez, & Merillon, 2009). Each method relates to the generation of a different radical, acting through a variety of mechanisms. In this sense, the antioxidant activity of different extracts was evaluated in four different *in vitro* models (Table 9.1). The four assays gave comparable results for the antioxidant activity measured in extracts. In general, violet petals were the most active with three types of solvent employed.

Significant differences in the antioxidant activity of the extracts were detected depending on the extraction solvent. The 50% ethanol extract was the most active in the yellow and red petals (651.51 µg TE/g and 954.16 µg TE/g, respectively) in DPPH assay. However, the better extraction solvent for violet petals was water (1026.65 µg TE/g). Similar results

were revealed in ABTS, ORAC and FRAP assays (Table 9.1). There was a strong correlation between the values obtained with DPPH, ABTS ORAC and FRAP. Similarly, Ou et al. (2002) reported high correlation of antioxidant activity between the FRAP and ORAC techniques in blueberry fruit, and Awika et al. (2003) observed as well high correlation between ABTS, DPPH, and ORAC among sorghum and its products.

TPC, TFC and TAC of all extracts showed solvent and pH dependence. Variations in the contents of various extracts were attributed to the polarities of the compounds present. The TPC values were in the range from 120.56 mg GAE/g to 465.76 mg GAE/g. In all cases, the TPC content was higher when the extraction solvent was 50% ethanol, followed by 50% ethanol in acid media and water. The TPC values of the violet (465.76 mg GAE/g) and red (464.17 mg GAE/g) petals were similar and significantly higher than in the yellow (287.51mg GAE/g) one. Gallego et al. (2013) reported that TPC values for thyme and lavender flowers were 288 and 52 mg GAE/g, respectively. This values are similar or lower than found in the current study. The good correlations between TPC and DPPH radical scavenging activity (r^2=0.6727), $ABTS^+$ radical scavenging activity (r^2=0.8851), ORAC method (r^2=0.9234) and FRAP assay (r^2=0.7681) values confirmed that mainly the phenols were responsible for the antioxidant activity of the extracts. These results were in agreement with previous studies of edible and wild flowers from China like *Rosa rugosa, Limonium sinuatum or Pelargonium hortorum* (Li et al., 2014).

TFC was also determined and values ranging from 28.20 mg RE/g to 198.57 mg RE/g were found. These results confirmed the previous literature reports indicating that flavonoids represent the main group of phenolic compounds in pansy (Vukics et al., 2008 b). The highest TFC value was obtained using water as an extraction solvent in the three colours: yellow (55.91 mg RE/g), red (83.58 mg RE/g) and violet (198.57 mg RE/g), followed by 50% ethanol and 50% ethanol in acid media. The study also showed a high and positive correlation between TFC content and DPPH radical scavenging activity (r^2=0.6136), $ABTS^+$ radical scavenging activity (r^2=0.8400), ORAC method (r^2=0.7941) and FRAP assay (r^2=0.8342).

Acidified media showed influence on the anthocyanin content and the extraction efficiency was higher at lower pH values. The higher TAC values were seen in violet (38.74 mg ME/g), followed by red (28.72 mg ME/g) and finally yellow (13.98 mg ME/g) petals. However, low correlations were found between total anthocyanin content and

antioxidant activity. Therefore, good correlation suggested that phenolic compounds, mainly of flavonoid type, play an important role as antioxidants. Variations in the antioxidant capacity of different extracts may be attributed to differences in their individual phenolic contents.

Significant advantages over other chromatographic techniques, such as the potential of being used for qualitative and quantitative analysis with high sensitivity and specificity, had made HPLC-MS a widespread and extremely useful method for phytochemical analysis. This technique combines HPLC, a powerful tool for complex mixture separation with mass spectrometry which enables reliable determination of the molecular weight and characterization of ionised compounds.

Table 9.1 Polyphenol, flavonoid, anthocyanin content and antioxidant activity of different *Viola wittrockiana* extracts.

	Yellow			Red			Violet		
	H_2O	50% EtOH	50% EtOH (pH=2)	H_2O	50% EtOH	50% EtOH (pH=2)	H_2O	50% EtOH	50% EtOH (pH=2)
TPC (mg/g)	120.56 ±4.48^{d}	287.51 ±4.05^{c}	257.07 ±7.59^{c}	419.28 ±4.75^{b}	464.17 ±8.06^{a}	352.56 ±10.04^{b}	395.90 ±6.24^{b}	465.76 ±9.72^{a}	442.52 ±14.35^{b}
TFC (mg/g)	55.91 ±1.19^{c}	35.79 ±2.43^{e}	28.20 ±0.07^{e}	83.58 ±1.60^{b}	46.11 ±0.40^{d}	42.84 ±0.14^{d}	198.57 ±3.27^{a}	45.35 ±0.40^{d}	41.20 ±1.85^{d}
TAC (mg/g)	4.11 ±0.13^{e}	9.66 ±0.42^{d}	13.98 ±0.95^{d}	21.50 ±0.52^{c}	23.50 ±0.68^{c}	28.72 ±0.92^{b}	19.24 ±0.75^{c}	17.64 ±0.27^{c}	38.74 ±5.12^{a}
DPPH (µmol/ g)	357.83 ±1.20^{d}	651.51± 4.31^{c}	552.32 ±1.80^{c}	928.19 ±2.26^{b}	954.16 ±2.61^{b}	555.81 ±3.97^{c}	1026.65 ±1.33^{a}	961.88 ±1.64^{b}	652.31 ±2.76^{c}
ABTS (µmol/ g)	519.79 ±2.50^{e}	998.30± 2.59^{c}	906.06 ±6.47^{d}	1100.00 ±2.04^{c}	1178.23 ±4.82^{b}	1098.39 ±1.83^{c}	1482.78 ±1.17^{a}	1103.94 ±2.26^{b}	1200.00 ±6.31^{b}
ORAC (µmol/ g)	791.90 ±0.31^{e}	2232.47± 1.11^{d}	2152.28 ±2.69^{d}	3057.20 ±0.58^{c}	3590.09 ±2.57^{b}	2758.74 ±1.42^{c}	3863.65 ±0.63^{a}	3744.59 ±0.93^{b}	3640.80 ±0.83^{b}
FRAP (µmol/ g)	418.34 ±3.94^{d}	503.60± 4.52^{c}	228.39 ±6.03^{e}	880.69 ±2.04^{b}	953.30 ±2.75^{a}	719.45 ±3.30^{b}	989.30 ±0.60^{a}	758.66 ±1.17^{b}	400.72 ±1.63^{d}
F1 (mg/g)	3.63 ±0.04^{b}	2.85 ±0.02^{b}	-	4.49 ±0.12^{a}	-	1.54 ±0.35^{c}	-	-	-
F2 (mg/g)	-	-	-	10,18 ±0.89^{a}	8,94 ±0.87^{a}	13,16 ±2.02^{a}	-	-	-
F3 (mg/g)	5.60 ± 0.14^{d}	3.68 ±0.05^{d}	7.32 ±0.11^{d}	30.14 ±2.33^{b}	17.27 ±1.21^{c}	17.36 ±1.67^{c}	107.96 ±4.36^{a}	26.17 ±1.87^{b}	21.30 ±2.79^{b}
F4 (mg/g)	-	-	-	-	-	-	37.66 ±1.88^{a}	6.76 ±0.42^{b}	8.80 ± 0.61^{b}
F5 (mg/g)	1.80 ±0.02^{d}	1.43 ±0.01^{d}	1.72 ±0.15^{d}	3.56 ±0.36^{c}	1.84 ± 0.02^{d}	1.72 ±0.03^{d}	32.34 ±2.45^{a}	7.38 ±0.70^{b}	5.24 ± 0.29^{b}
F6 (mg/g)	3.09 ±0.01^{b}	2.44 ±0.09^{c}	4.75 ±0.07^{b}	6.71 ±1.43^{a}	3.30 ±0.07^{b}	3.62 ±0.06^{b}	8.55 ±0.11^{a}	2.36 ±0.03^{c}	1.92 ± 0.02^{c}
F7 (mg/g)	40.81 ±2.87^{a}	24.12 ±1.49c	10.98 ±0.99^{d}	33.61 ±3.01^{b}	19.55 ± 2.01^{d}	16.18 ±1.02^{d}	4.36 ±0.09^{e}	1.37 ±0.01^{e}	1.26 ± 0.01^{e}
F8 (mg/g)	-	-	-	2.65 ±0.21^{a}	-	1.80 ±0.01^{a}	-	-	-
F9 (mg/g)	-	-	-	1.88 ±0.13^{a}	-	-	-	-	-
F10 (mg/g)	-	-	-	0.76 ±0.01^{a}	-	-	-	-	-
Total	*54.94± 1.16*^{c}	*34.53 ±1.58*^{e}	*24.84±1 .32*^{e}	*79.34 ±3.49*^{b}	*42.01 ±2.31*^{d}	*40.66 ±2.16*^{d}	*190.87 ±5.09*^{a}	*44.05 ±1.03*^{d}	*38.53 ± 1.72*^{d}
A1 (mg/g)	2.96 ±0.02^{d}	4.58 ±0.11^{c}	4.69 ±0.20^{c}	7.47 ±1.05^{b}	8.80 ±0.92^{b}	11.40 ±1.74^{a}	7.01 ±0.77^{b}	8.56 ±0.46^{b}	11.58 ±1.02^{a}
A2 (mg/g)	0.40 ±0.01^{f}	2.08 ±0.06^{e}	1.99 ±0.13^{e}	5.17 ±1.00^{c}	6.89 ±0.54^{b}	7.72 ±0.83^{b}	3.49 ±0.04^{d}	0.78 ±0.00^{f}	9.89 ±1.01^{a}
A3 (mg/g)	0.65 ±0.01^{e}	2.83 ±0.09^{c}	4.39 ±0.27^{b}	5.75 ±0.99^{b}	4.39 ±0.08^{b}	7.25 ±0.05^{a}	3.74 ±0.22^{c}	4.27 ±0.70^{b}	7.74 ±0.81^{a}
A4 (mg/g)	-	-	2.72 ±0.34^{c}	3.03 ±0.72^{c}	3.62 ±0.17^{c}	4.74 ±0.34^{b}	4.88 ±0.31^{b}	3.67 ±0.52^{c}	9.07 ±1.00^{a}
Total	*4,02 ±0.57*^{e}	*9.49 ±1.04*^{d}	*13.81 ±1.94*^{c}	*21.43 ±2.75*^{b}	*23.70 ±2.13*^{b}	*31.11 ± 2.52*^{a}	*19.13 ±1.37*^{b}	*17.30 ±1.51*^{b}	*38.29 ±2.71*^{a}

Data expressed as means ± SD of triplicate analyses. Values in the same line with different letters present significant differences $p < 0.05$.

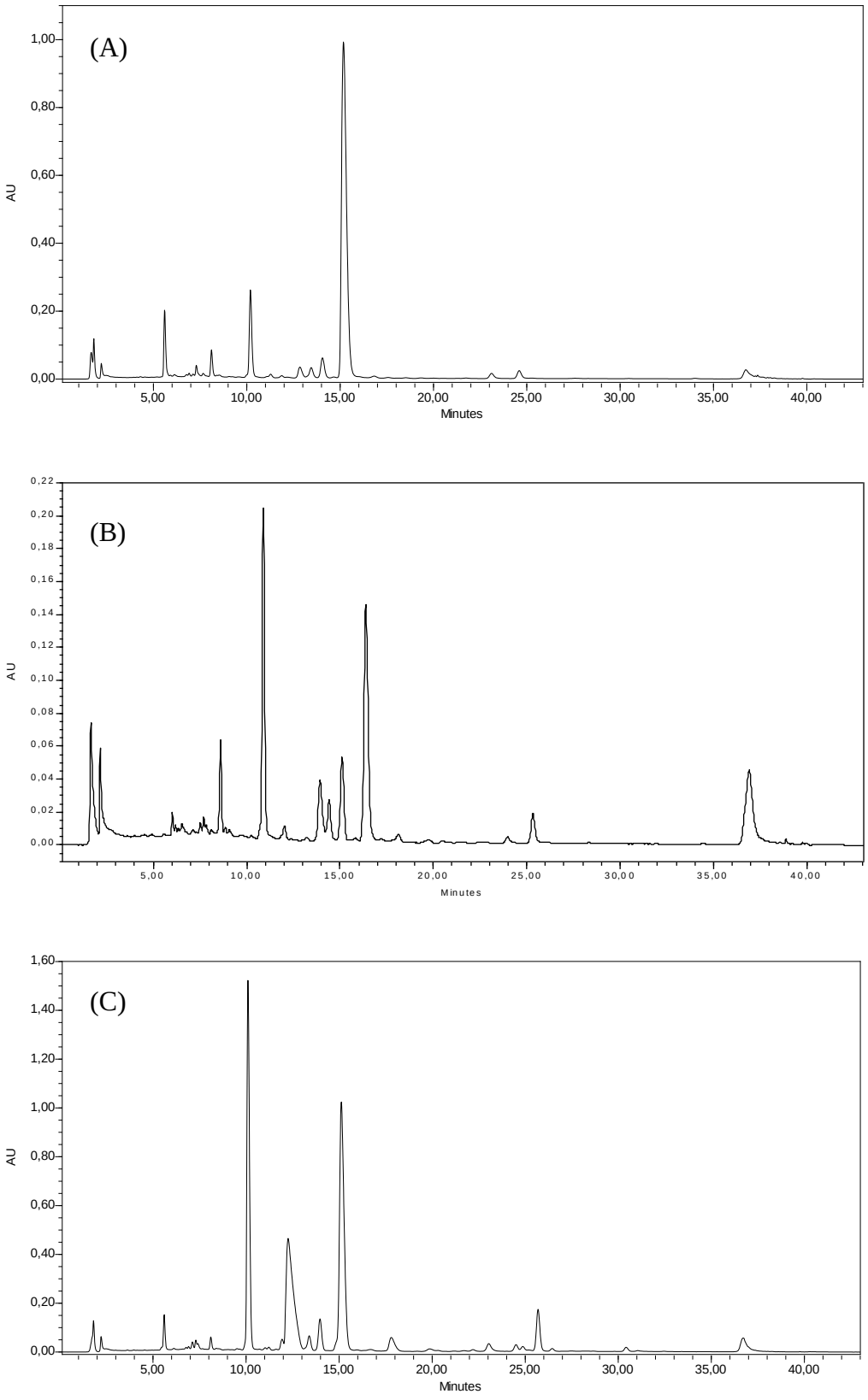
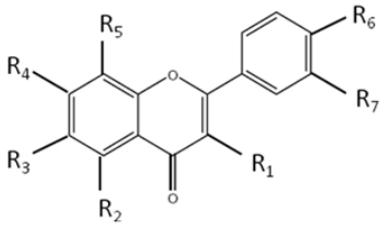


Figure 9.1 Chromatographic profiles, acquired at 355 nm, of *V. wittrockiana* extracts (A: yellow, B: red, C: violet colour).

Components of red, yellow and violet garden pansy, extracted with three different solvents were identified and quantified by comparing retention times, ultraviolet and mass spectra, and mass fragmentation data with those described in existing literature. In total, 14 constituents were identified by HPLC-DAD-ESI-MS. In Fig. 9.1, the HPLC-DAD chromatograms of the yellow, red and violet petals are presented. Data concerning the identification of the peaks are shown in Table 2, where the retention time, UV-Vis absorptions and electrospray ionisation mass spectrometry in negative and positive ion mode of all the compounds detected are reported.

HPLC-DAD-ESI-MS analyses evidenced the presence of 10 flavonoids (Table 9.2, Fig.9.2). Their UV spectra exhibited two major absorption maxima in the region of 240-400 nm. HPLC-MS and HPLC-UV data indicated the presence of complex flavonol and flavone glucosides. Compounds **F1**, **F3**, and **F9** were derivatives of quercetin, whereas **F5**, **F6, F7**, **F8** and **F10** revealed the presence of apigenin. Fragmentation patterns of compounds **F2** and **F4** suggested the presence of kaempferol and luteolin derivatives, respectively.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
F1	-ORha-Rha-Glu	-OH	-H	-OH	-H	-OH	-OH
F2	-ORha-Rha-Glu	-OH	-H	-OH	-H	-OH	-H
F3	-O-Glu-Rha	-OH	-H	-OH	-H	-OH	-OH
F4	-H	-OH	-Rha	-OH	-Glu	-OH	-OH
F5	-H	-OH	-Rha	-OH	-Glu	-OH	-H
F6	-H	-OH	-Glu	-OH	-Glu	-OH	-H
F7	-H	-OH	-H	-OH	-Glu	-OH	-H
F8	-H	-OH	-Glu	-OH	-H	-OH	-H
F9	-OGlu	-OH	-H	-OH	-H	-OH	-OH
F10	-H	-OH	-H	-OGlu	-H	-OH	-H

Figure 9.2 Chemical structures of flavonoids identified from *Viola wittrockiana*.

Table 9.2 Retention time, UV-vis absorption data, MS fragmentation and name of the main compounds detected in *Viola wittrockiana.*

No.	Rt	UV (nm)	*m/z*	Negative	Positive	Identification	Literature reference
F1	5.6	255.6, 352.9	756	755 $[M-H]^-$, 593 [M-H-Glu]$^-$, 447 [M-H-Glu-Rha]$^-$, 301 $[A-H]^-$	779 $[M+Na]^+$, 757 $[M+H]^+$, 303 $[A+H]^+$	Quercetin-3-*O*-di-rhamnosyl-glucoside	Karioti et al. (2011)
A1	6.1	288, 312sh, 529	919	935 $[M-2H+H_2O]^-$,755 [M-Glu]$^-$, 609 [M-Glu-Cm]$^-$, 563 [M-Glu-Cm-CH_2]$^-$	757 [M-Cm]$^+$, 465 [M-Cm-Rha-Glu]$^+$, 303 $[A]^+$,	Delphinidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside (violanin)	Hase et al. (2005), Karioti et al. (2011), Zhang et al. (2011)
A2	8.0	312sh, 528	933	949 $[M-2H+H_2O]^-$, 769 [M-Glu]$^-$, 623 [M-Glu-Cm]$^-$, 577 [M-Glu-Cm-CH_2]$^-$	761 [M-Cm]$^+$, 479 [M-Cm-Rha-Glu]$^+$, 317 $[A]^+$,	Petunidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside	Zhang et al. (2011)
F2	8.1	266, 344	740	739 $[M-H]^-$, 577 [M-H-Glu]$^-$, 431 [M-H-Glu-Rha]$^-$, 285 $[A-H]^-$	763 $[M+Na]^+$, 741 $[M+H]^+$, 287 $[A+H]^+$	Kaempferol-3-*O*-di-rhamnosyl-glucoside	Karioti et al. (2011)
A3	8.4	312sh, 520	903	919 $[M-2H+H_2O]^-$, 739 [M-Glu]$^-$, 593 [M-Glu-Cm]$^-$, 547 [M-Glu-Cm-CH_2]$^-$	741 [M-Cm]$^+$, 449 [M-Cm-Rha-Glu]$^+$, 287 $[A]^+$	Cyanidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside	Zhang et al. (2011)
F3	10.2	255.6, 354.1	610	609 $[M-H]^-$, 463 [M-H-Rha]$^-$, 301 $[A-H]^-$	633 $[M+Na]^+$, 611 $[M+H]^+$, 303 $[A+H]^+$	Quercetin-3-*O*-rhamnosyl-glucoside (rutin)	Karioti et al. (2011), Papp et al. (2004), Vukics et al. (2008b,c)
A4	10.7	311sh, 535	947	963 $[M-2H+H_2O]^-$, 783 [M-Glu]$^-$, 637 [M-Glu-Cm]$^-$, 591 [M-Glu-Cm-CH_2]$^-$	775 [M-Cm]$^+$, 493 [M-Cm-Rha-Glu]$^+$, 331 $[A]^+$,	Malvidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside	Zhang et al. (2011)
F4	12.4	255.6, 352.9	626	625 $[M-H]^-$, 463 [M-H-Rha]$^-$, 447 [M-H-Glu]$^-$, 285 $[A-H]^-$	649 $[M+Na]^+$, 627 $[M+H]^+$, 287 $[A+H]^+$	Luteolin-6-*C*-rhamnosyl-8-*C*-glucoside	Present study
F5	13.5	266.2, 341.0	610	609[M-H]$^-$, 447 [M-H-Rha]$^-$, 431 [M-H-Glu]$^-$, 269 $[A-H]^-$	649 $[M+Na]^+$, 611 $[M+H]^+$, 271 $[A+H]^+$	Apigenin-6-*C*-rhamnosyl-8-*C*-glucoside (violanthin)	Vukics et al. (2008b,c)
F6	14.3	271.0, 335.0	626	625 $[M-H]^-$, 447 [M-H-Glu]$^-$, 269 $[A-H]^-$	649 $[M+Na]^+$, 627 $[M+H]^+$, 271 $[A+H]^+$	Apigenin-6,8-di-*C*-glucoside (vicenin-2)	Vukics et al. (2008a)
F7	15.2	266.6, 354,1	448	447 $[M-H]^-$, 269 $[A-H]^-$	471 $[M+Na]^+$, 449 $[M+H]^+$, 271 $[A+H]^+$	Apigenin-8-*C*-glucoside (orientin)	Vukics et al. (2008a)
F8	17.8	266.4, 348.7	448	447 $[M-H]^-$, 269 $[A-H]^-$	471 $[M+Na]^+$, 449 $[M+H]^+$, 271 $[A+H]^+$	Apigenin-6-*C*-glucoside (isoorientin)	Vukics et al. (2008a)
F9	23.0	255.6, 348.1	464	463 $[M-H]^-$, 301 $[A-H]^-$,	487 $[M+Na]^+$, 465 $[M+H]^+$, 303 $[A+H]^+$	Quercetin-3-*O*-glucoside (isoquercitrin)	Karioti et al. (2011), Papp et al. (2004)
F10	25.6	265.1, 331.4	432	431 $[M-H]^-$, 269 $[A-H]^-$	455 $[M+Na]^+$, 433 $[M+H]^+$, 271 $[A+H]^+$	Apigenin-7-*O*-glucoside (apigetrin)	Karioti et al. (2011)

Molecular masses were higher than 400 Da and the characteristic ions of sugar losses (146 Da and 162 Da for deoxyhexose and hexose units, respectively) indicated the presence of one or more sugar units in their structure.

The high retention times of 15.2, 17.8, 23.0 and 25.6 min of less polar compounds **F7**, **F8**, **F9** and **F10** in combination with their mass data and literature references indicated the presence of one sugar unit. The MS spectra of peaks **F9** and **F10** showed the lack of the fragment ions characteristic of sugar losses (162 Da) for glucose units, which suggested that both compounds were flavonoid *O*-glucosides. Similarly, compounds **F7** and **F8** are flavonoid C-glucosides $[M-H-178]^-$. These results suggested the identity of the compounds as orientin (**F7**), isoorientin (**F8**), isoquercitrin (**F9**) and apigetrin (**F10**), which is compatible with literature references for *Viola wittrockiana* and other species of *Viola* (Karioti, Furlan, Vincieri, & Bilia, 2011; Papp et al., 2004).

Fragmentation patterns for compounds **F3**, **F4**, **F5** and **F6** showed the presence of a second sugar. Compound **F3** was identified, based on the presence of molecular ion at *m/z* 610 $[M]^+$ in MS spectra, UV spectra and several literature references (Karioti et al., 2011, Papp et al., 2004, Vukics et al., 2008b, 2008c) as rutin, compound **F5** as violanthin and compound **F6** as vicenin-2.

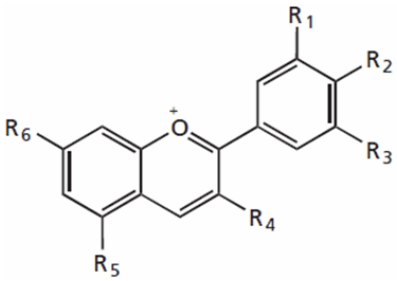
Compound **F4** was identified as luteolin-6-*C*-rhamnosyl-8-*C*-glucoside. The structural elucidation was determined first of all on the basis of the UV spectra (λ_{max}=255.6, 352.9 nm). Furthermore, the MS spectrum (*m/z* 626 $[M]^+$, 625 $[M-H]^-$, 463 $[M-H-Glu]^-$, 447 $[M-H-Rha]^-$, 285 $[A-H]^-$) was similar to that of the compound **F5**. The difference of 16 u in the molecular ion was assigned to one additional hydroxyl group at C3´ of the ring B of the flavonoid. Since this constituent was observed for the first time in *Viola sp*., it would be necessary to characterize it completely by 1D and NMR spectroscopy after isolation and purification.

On the other hand, compounds **F1** and **F2**, at earlier retention times (5.6 and 8.1 min), displayed a molecular ions at *m/z* 756 and 740 respectively, due to the presence of a third sugar. Compound **F1** was identified as quercetin-3-*O*-rhamnorhamnoglucoside and compound **F2** as kaempferol-3-*O*-rhamnorhamnoglucoside, based on the data obtained from the literature from *V. odorata* (Karioti et al., 2011).

The petal of red and violet colour indicated the presence of anthocyanins, which are glycosides and acylglycolides of anthocyanidins, according to the hydroxyl or methoxyl

substitutions in the basic skeleton. In this type of compounds, acid media are generally preferred in HPLC analyses, as they enhance anthocyanin stabilisation. In this case, the possible presence of labile flavonoids with more than two saccharides, as well as the fact that under acid conditions hydrolysis of labile acyl groups may occur, did not permit to conduct the analyses in pH lower than 2.5.

Four anthocyanins (**A1**, **A2**, **A3** and **A4**) were tentatively identified by comparing retention time, MS and UV-vis spectrum (Fig. 9.3). All of them showed a typical chromatogram of the anthocyanins with selective wavelength (500–550nm absorption). The chromatographic and spectral data are summarised in Table 2. These identifications were confirmed by the scientific literature (Karioti et al., 2011).



	R_1	R_2	R_3	R_4	R_5	R_6
A1	-OH	-OH	-OH	-O-Glu-*p*-CmRha	-OGlu	-OH
A2	-OH	-OH	$-OCH_3$	-O-Glu-*p*-CmRha	-OGlu	-OH
A3	-OH	-OH	-H	-O-Glu-*p*-CmRha	-OGlu	-OH
A4	$-OCH_3$	-OH	$-OCH_3$	-O-Glu-*p*-CmRha	-OGlu	-OH

Figure 9.3 Chemical structures of anthocyanins identified from *Viola wittrockiana.*

Anthocyanins derived from aglycones cyanidin, delphinidin, petunidin and malvidin were identified, according to the presence of a series of fragment signals at *m/z* 287, 303, 317 and 331 in MS spectra, respectively. The shoulder absorption at 290-340 nm showed that compounds were acylated with aromatic acids (Wrolstad, Durst, & Lee, 2005).

Violanin (**A1**) was assigned to a peak with retention time of 6.1 min and showed a characteristic pseudomolecular ion in the positive ion mode at *m/z* 919 and a peak at *m/z* 303 (corresponding to the delphinidin aglycone). UV maximum was at 312 nm due to the presence of the coumaroyl moiety. Other characteristic ions at *m/z* 757 $[M-162]^-$, *m/z* 465

$[M-308-146]^-$, were observed and attributed to the loss of coumaroyl and coumaroyl–rutinosyl moieties.

Peaks **A2**, **A3** and **A4** had similar mass spectra patterns as **A1**. Based on their retention times and literature references, they were identified as petunidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside (t_R=8.0 min), cyanidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside (t_R=8.4 min) and malvidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside (t_R=10.7 min), respectively. Several anthocyanins were detected at earlier times but the content was very low and their identification was impossible.

In Table 9.1, the results of the quantitative analyses are reported. Concerning flavonoids, rutin (**F3**), violanthin (**F5**), vicen-2 (**F6**) and orientin (**F7**) were presented in all extracts. The major flavonoid found was **F3** (107.96±4.36 mg/g), followed by **F5** (32.34±2.45 mg/g) in aqueous extract of violet petals. The same results had been presented by Vukics et al. (2008c). Moreover, Goncalves et al. (2012) reported a similar content of rutin in *Viola tricolor* flowers in ethyl acetated and butanol fractions (33.70 and 143.57 mg/g, respectively).

Luteolin-6-*C*-rhamnosyl-8-*C*-glucoside (**F4**) was detected only in violet pansy. The content was 4-6-fold higher in aqueous (37.66±1.88 mg/g) than 50% ethanol (6.76±0.42 mg/g) or 50% ethanol in media acid (8.80±0.61 mg/g) extract. In the same way, kaempferol-3-*O*-di-rhamnosyl-glucoside (**F2**), isoorientin (**F8**), isoquercetrin (**F9**) and apigetrin (**F10**) appeared only in red pansy. The good correlation found between quercetin and luteolin derivatives (**F1**, **F3**, **F4**, **F9**) and antioxidant activity assays (r^2>0.9) suggests that these compounds may be partly responsible for this activity. This confirms the importance of the presence of hydroxyl groups in *ortho* position in relation to this activity, which has previously been described by numerous authors (Casado et al., 2011).
The four previously identified anthocyanins (**A1**, **A2**, **A3**, **A4**) were detected and quantified in all extracts analyzed, with the exception of malvidin-3-(4´´-*p*-coumaroyl)-rutinoside-5-glucoside (**A4**), which could not be quantified in water and 50% ethanol extract of yellow pansy (Table 9.1).

In all extracts anthocyanin content was pH dependent, and therefore the best results were obtained when the solvent for extraction was 50% ethanol in acid media.
Violanin (**A1**) was the major anthocyanin in petals of three different colours, highlighting its high content in red (11.40 mg/g) and violet (11.58 mg/g) in comparison with yellow

(4.69 mg/g) colour. The good correlation found between violanin (**A1**) and antioxidant activity assays (r^2=0.877) suggests that this compound was also involved in the antioxidant activity. Delphinidin derivates were reported to have relatively strong antiradical activity among various anthocyanins (Kahkonen & Heinonen, 2003).

Anthocyanins are a class of compounds responsible for red, blue and violet colours of plants. However, this class of compounds is highly unstable and easily susceptible to degradation. The stability of anthocyanin color can be improved by copigmentation, where the anthocyanin molecule reacts with other natural plant components directly or through weak interactions, resulting in an enhanced and stabilized color (Di Meo, Sancho Garcia, Dangles, & Trouillas, 2012, Deng et al., 2013). A wide range of different molecules has been found to act as copigments. The most studied group of copigments is flavonoids, of which flavones, flavonols, flavanones, and flavanols have been under profound examination. Several complex formations (Fig. 9.4) through intermolecular interactions between delphinidin derivatives and quercetin derivatives have been described (Osawa, 1982).

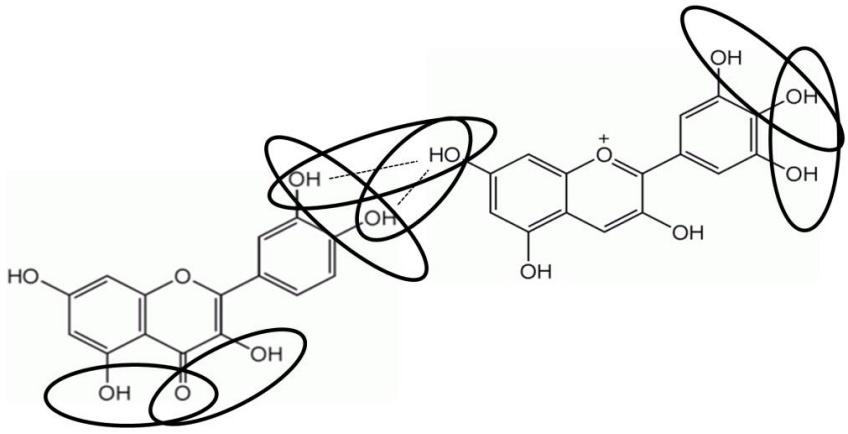


Figure 9.4 *ortho*-dihydroxyphenil moiety and complex formations through intermolecular interactions between delphinidin and quercetin.

Therefore, this copigmentation phenomenon could demonstrate the high antioxidant activity detected in aqueous extract of violet pansy, taking into consideration several factors, such as: i) high content in rutin and violanin (quercetin and delphinidin derivatives, respectively), ii) the *ortho*-dihydroxyphenil moiety in both types of compounds, iii) complex between anthocyanins and flavonoids, which are soluble in water solution.

9.4 Conclusion

This study has proved the antioxidant activity of extracts from pansy. The extracted amount of phenolics, flavonoids and anthocyanins, as well as their antioxidant activity, depend on both the extraction solvent and petal colour. In this way, the use of a solution of ethanol 50% results in the highest quantity of phenolics. The phenolic content in violet and red petals was similar and significantly higher than in yellow petals. Flavonoids represented the main group of phenolic compounds in pansy. The flavonoid content of violet petals was significantly higher than that of red and yellow material. Acidified media showed influence on the anthocyanin content and the extraction was more efficient at lower pH values. Chromatographic analysis with UV spectra recorded on-line gave useful complementary information and HPLC coupled to mass spectrometry (LC-MS) provided additional structural information on the potentially active metabolites. In total, 14 constituents were identified by HPLC-DAD-ESI-MS, 10 of them were flavonoids (quercetin and apigenin derivatives, mainly) and 4 anthocyanins. Concerning flavonoids, rutin, violanthin, vicen-2 and orientin were presented in all extracts. The major flavonoid found was rutin, followed by violanthin in aqueous extract of violet petals. Violanin (delphinidin glucoside) was the major anthocyanin in the three pansy colours, with the highest values measured in red and violet petals. In conclusion, the copigmentation phenomenon between violanin and rutin evidences the high antioxidant activity detected in aqueous extract of violet pansy. Our results indicate that aqueous extracts of violet pansy can be used in functional foods as potential radical scavenging agents.

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10 Conclusion and objectives of future research

10.1 Conclusion

Tara (*Caesalpinia spinosa*) pods are an outstanding source of phenolic compounds and flavonoids, which can be extracted to a different extent using water or ethanol/water mixtures. The extracted amount of phenolics and flavonoids, as well as their antioxidant activity, depend on both the solvent and the extraction method. For instance, the use of 75% aqueous ethanol in a 1-h ultrasonic process allows achieving the greatest quantity of phenolics (0.467 g gallic acid equivalent (GAE)/g DW) and provides the best antioxidant activity as measured by the $ABTS^{+}$ and AAPH radicals (10.17 and 4.29 mmol Trolox equivalents (TE)/g DW, respectively). The highest amount of flavonoids is extracted applying a 24-h maceration in cold water, and 24-h cold maceration in 50% aqueous ethanol leads to the extract with the highest antioxidant activity as assessed through the FRAP method.

The lyophilized ethanolic (75%) and aqueous extracts were added to a model food system (oil-in-water emulsion) and the oxidative stability was studied during storage at 38°C. The addition of 48 mg/L of ethanol extract to the emulsion with 10% of oil delayed oxidation to the same extent as 17.8 mg/L of Trolox, while water extract was only effective in the early stages of the oxidation process. The oxidative process in the emulsion can be assessed either by measuring the peroxide value or the pH, since both parameters are strongly related by an exponential-type equation.

In addition, the dried pods from tara can be successfully used to decrease lipid oxidation and improve the shelf life and colour stability of cooked model meat systems. Tara at 0.02% was as effective as BHA at the same concentration in retarding lipid oxidation in cooked pork products during storage. Results showed that redness increased upon the addition of tara. Specifically, 0.02% of tara was effective in keeping red colour of meat products stored under illumination at 4ºC for 48 h.

The results of antimicrobial activity of the extract of tara pods show that it can also act as an inhibitor against *Staphylococcus aureus, Shigella sonnei, Micrococcus luteus* and *Proteus mirabilis.* The gram-positive *S. aureus* was the most sensitive organism to tara pod extract and the minimum inhibitory concentration (MIC) was found to be

6.25mg/mL. On the other hand, tara pod extract showed growth promoting effect on the common probiotic bacterium *L. plantarum.*

The ethanolic extract (50%) from *Perilla frutescens* leaves and stalks contained 22.67 ± 0.52 mg GAE/g DW of phenolics and 2.90 ± 0.07 mg CE/g DW of flavonoids. The measurement of scavenging capacity against the ABTS+ radical, the oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant power (FRAP) were 65.03 mg TE/g DW, 179.60 mg TE/g DW and 44.46 mg TE/g DW, respectively. The presence of caffeic and rosmarinic acids was determined by HPLC and the contents of phenolic acids were 0.51 mg/g dry weight (DW) and 2.29 mg/g DW, respectively. Both compounds can induce beneficial and health promoting effects. *P. frutescens* extracts in 10% sunflower oil-in-water emulsions also showed good antioxidant properties during storage at 32±1 ºC. Lyophilized perilla extract at 320 mg/L was as effective as BHA at 20 mg/L in slowing down the formation of hydroperoxides, thiobarbituric acid reactive substances and hexanal content.

The total polyphenols and flavonoids in extracts of *A. annua* leaves obtained with 50% aqueous ethanol were 23.36 ± 0.92 mg GAE/g DW and 2.68 ± 0.07 mg CE/g DW, respectively. The measurement of the scavenging capacity against $ABTS^{+}$ radical, ORAC and the FRAP were 314.99 µmol TE/g DW, 736.26 µmol TE/g DW and 212.18 µmol TE/g DW, respectively. The main compounds detected by LC-MS in extract of *A. annua* were caffeic acid, rutin and apigenin. In addition, *A. annua* extracts showed good antioxidant properties in 10% sunflower oil-in-water emulsions during prolonged storage (45 days) at 32±1 ºC. Artemisia extract at 2 g/L was as effective as butylated hydroxyanisole (BHA) at 0.02 g/L in slowing down the formation of hydroperoxides and thiobarbituric acid reactive substances.

The extracts from garden pansy (*Viola wittrockiana*) are an outstanding source of phenolics, flavonoids and anthocyanins. The extracted amount of phenolic compounds as well as their antioxidant activity, depend on both the solvent (water, 50% ethanol and 50% ethanol at pH 2) and petal colour (yellow, red or violet). The use of a 50% solution of ethanol results in the highest quantity of phenolics. The phenolic content in the violet and red petals was similar and significantly higher than in the yellow petals. Flavonoids represented the main group of phenolic compounds in pansy. The flavonoid content of the violet petals was significantly higher than that of red and yellow material. Acidified

media showed influence on the anthocyanin content and the extraction was more efficient at lower pH values. Chromatographic analysis with UV spectra and HPLC coupled to mass spectrometry (LC-MS) provided additional structural information on the potentially active metabolites. In total, 14 constituents were identified by HPLC-DAD-ESI-MS, 10 of them were flavonoids (quercetin and apigenin derivatives, mainly) and 4 anthocyanins. Concerning the flavonoids, rutin, violanthin, vicen-2 and orientin were present in all extracts. The major flavonoid found was rutin, followed by violanthin in aqueous extract of violet petals. Violanin (delphinidin glucoside) was the major anthocyanin in the three pansy colours, with the highest values measured in the red and violet petals.

The present study has demonstrated the potential of using plant materials as natural ingredient in the development of food functional products which show potential health benefits. Especially, tara (*C. spinosa*) pods are suitable for use as natural food additives for lipid stabilization in processed foods.

10.2 Future work

The future research should include further work aimed at optimizing the extraction procedures (such as microwave-assisted extraction, supercritical fluid extraction or high hydrostatic pressure extraction). In addition, further studies should investigate the application of novel antioxidant mixtures to develop new products of novel structures using techniques such as encapsulation or nanotechnology.

Addition of plant material can impact the colour and flavour of the food products. Therefore, a sensory evaluation of these products needs to be conducted. Moreover, new group of antioxidants which has been isolated from non-food plants should be studied under the same conditions as synthetic antioxidants.

In the present study, the main purpose of the microbiological analysis was to test the tara pod extracts *in vitro*. Further studies should investigate antimicrobial or probiotic activities of tara pod extracts in model food systems (such as meat or dairy products) and *in vivo*.

11 Anex

The next publications and presentations have resulted from the research work presented in this thesis:

Publications

Skowyra, M., Falguera, V., Gallego, G., Peiró, S. & Almajano, M.P. (2014) Antioxidant properties of aqueous and ethanolic extracts of tara (*Caesalpinia spinosa*) pods *in vitro* and in model food emulsions. *J. Sci. Food Agric. 94*, 911-918.

Skowyra, M., Falguera, V., Azman N.A.M., Segovia, F. & Almajano, M.P. (2014) The effect of *Perilla frutescens* extract on the oxidative stability of model food emulsions. *Antioxidants 3*, 38-54.

Skowyra, M., Gallego G., Segovia, F. & Almajano, M.P. (2014) Antioxidant properties of *Artemisia annua* extracts in model food emulsions. *Antioxidants, 3,* 116-128.

Skowyra, M., Tril, U., Salejda, A.M., Krasnowska, G., & Almajano, M.P. (2014) Effect of tara (*Caesalpinia spinosa*) pods on the quality and shelf-life stability of model meat systems during chilled storage. *Subbmitted Papper*.

Skowyra, M., Calvo, M.I., Gallego, G., Azman N.A.M. & Almajano, M.P. (2014) Characterization of phytochemicals in petals of different colours from *Viola x wittrockiana* Gams. and their correlation with antioxidant activity. *J. Agric. Science.* Vol. 6, No. 9. DOI:10.5539/jas.v6n9p93.

Conference Posters

Skowyra, M., Dávila Cozar, M. & Almajano, M.P. Antioxidant and antimicrobial activities of the pods of the tara tree (*Caesalpinia spinosa*). VI Congreso Nacional de Ciencia y Tecnologia de los Alimentos. Valencia, 8-10 June 2011.

Dávila Cozar, M., Skowyra, M. & Almajano, M.P. Antioxidant activity of *Perilla frutescens*. V International Conference on Polyphenols and Health. Sitges – Barcelona, 17-20 October 2011.

Publications that have not form part of this thesis:

Almajano, M.P. & Skowyra, M. (2011) Extract of *Allium cepa* as an alternative to synthetic antioxidants and preservatives in food. *Health Molecules and Ingredients,* 25-26 May 2011, Quimper, France.

Gallego, M.G., Gordon, M.H., Segovia, F. J., Skowyra, M. & Almajano, M.P. (2013) Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil-in-water emsulsions. *J. Am. Oil Chem. Soc., 90,* 1559-1568.

Azman N.A.M., Gordon, M.H., Skowyra, M., Segovia, F.J & Almajano, M.P. (2014) Antioxidant effects of *Gentiana Lutea* root in fresh beef patties stored under different atmospheres. *J. Sci. Food Agric*. DOI: 10.1002/jsfa.6878

Gallego, M.G., Gordon, M.H, Segovia, F.J., Skowyra, M. & Almajano, M.P. (2014) Antioxidant properties of *Caesalpinia decapetala* (roth) alston in oil-in-water emulsions and cooked sausages. *Subbmitted Papper.*