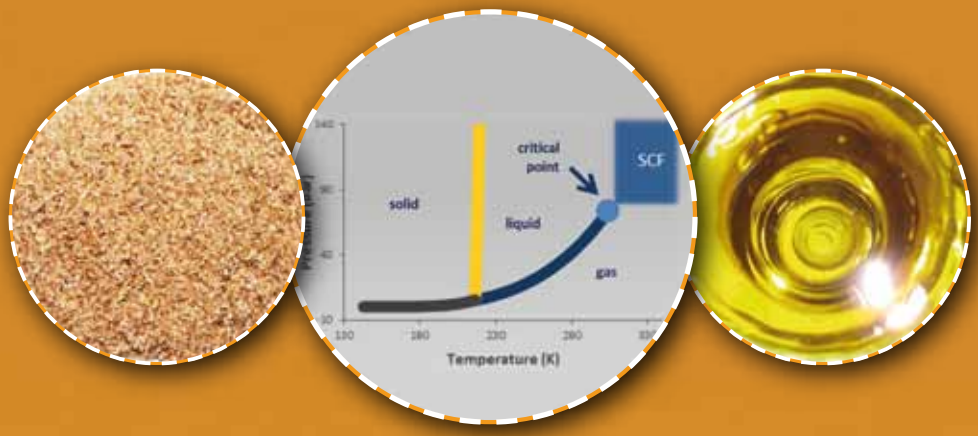
Sara Rebolleda Alonso

Wheat bran valorization by supercritical fluid extraction. Characterization, formulation and application of wheat bran oil



University of Burgos (Spain)
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Wheat bran valorization by supercritical fluid extraction. Characterization, formulation and application of wheat bran oil

Memoria que para optar al grado de Doctor por la Universidad de Burgos en el programa Avances en Ciencia y Biotecnología Alimentarias presenta
SARA REBOLLEDA ALONSO

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UNIVERSIDAD DE BURGOS

Departamento de Biotecnología y Ciencia de los Alimentos

Dña. MARÍA TERESA SANZ DÍEZ, Dña. SAGRARIO BELTRÁN CALVO, y Dña. MARÍA LUISA GONZÁLEZ SAN JOSÉ, Profesoras del Departamento de Biotecnología y Ciencia de los Alimentos de la Universidad de Burgos

CERTIFICAN:

Que la licenciada Dña. **SARA REBOLLEDA ALONSO** ha realizado bajo su dirección, el trabajo titulado **"Wheat bran valorization by supercritical fluid extraction. Characterization, formulation and application of wheat bran oil"**, cuyo título en castellano es: "Valorización de salvado de trigo mediante extracción con fluidos supercríticos. Caracterización, formulación y aplicación de aceite de salvado de trigo".

Considerando que dicho trabajo reúne los requisitos exigidos para ser presentado como Tesis Doctoral, expresan su conformidad con dicha presentación.

Y para que conste, firman el presente certificado,

En Burgos, a 29 de Abril de 2014

Fdo. M. Teresa Sanz Fdo. Sagrario Beltrán Fdo. M. Luisa González

UNIVERSIDAD DE BURGOS

Dña. **PILAR MUÑIZ RODRÍGUEZ**, coordinadora del programa de doctorado Avances en Ciencia y Biotecnología Alimentarias de la Universidad de Burgos,

CERTIFICA:

Que la memoria titulada "Wheat bran valorization by supercritical fluid extraction. Characterization, formulation and application of wheat bran oil" presentada por Dña. Sara Rebolleda Alonso, Licenciada en Ciencia y Tecnología de los Alimentos y Máster en Seguridad y Biotecnología Alimentarias, ha sido realizada en el Departamento de Biotecnología y Ciencia de los Alimentos bajo la dirección de las Dras. María Teresa Sanz Díez, Sagrario Beltrán Calvo, y María Luisa González San José, y en representación de la Comisión Académica del Programa de Doctorado, autoriza su presentación para ser defendida como Tesis Doctoral.

Y para que conste, y a efectos oportunos, firmo el presente certificado,

En Burgos, a 29 de Abril de 2014

Fdo. Pilar Muñiz Rodríguez
Coordinadora del Programa de Doctorado

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SUMMARY

Antioxidants addition is the most used strategy in the food industry to preserve food quality by retarding deterioration, rancidity or discoloration due to oxidation. Synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate are effective oxidation inhibitors and therefore, they are commonly used additives. Other commonly employed antioxidants are ascorbic acid and tocopherols, which can be both, natural or synthetic, and also sulphites. Sulphites functions include the inhibition of enzymatic and no enzymatic browning and also, the control of microbial growth. The main disadvantage that sulphites present is related to the allergic reactions in consumers in which they are involved, what has resulted in their strict regulation for its application into food products. The increasing consumers demand of natural products and the negative health effects caused by some additives are increasingly promoting the search of alternative natural antioxidants that can protect food products from oxidation and browning and their subsequent quality loss.

In recent years, different natural raw materials such as spices and herbs, tea and grape seed, among others, have been used as antioxidants sources. In this sense, wheat bran appears as a valuable source of antioxidants due to its content of bioactive compounds. Moreover, wheat bran is a readily available product since it is obtained as a by-product during the wheat milling process, mainly intended to animal feed.

The present research work is focused on the extraction of bioactive compounds from wheat bran by using a green technology, supercritical fluid extraction, which may contribute to both, the search of new natural antioxidants with potential uses into the food industry as well as the upgrading of a by-product from the wheat milling industry.

The commonly employed methods for the extraction of bioactive compounds are solvent extraction and hydrodistillation, and they usually involve the utilization of large amounts of solvents, long times and/or high temperatures. To overcome these disadvantages and to increase the extraction yield and extracts quality, different techniques have been developed in the last decades. The high process versatility without using organic solvents makes supercritical fluid extraction a suitable technique for the extraction of high value added products from a wide variety of matrices; in the particular case of this work, wheat bran.

The study of supercritical fluid extraction applied to wheat bran has been the aim of the first part of this work, where the evaluation of different parameters that influence the process has been carried out. These parameters include the extraction conditions and operation mode as well as other parameters related to the raw material characteristics.

In addition to the study of the extraction process, it has been also objective of this work the evaluation of the composition and quality of the extracted oil. Wheat bran is an important source of multiple components, many of which have been recognized as effective antioxidants. The characterization of the extracted oil and the evaluation of its antioxidant activity have been considered fundamental throughout the development of this work in order to recognize the potential use and applicability of the obtained wheat bran oil into the food industry, since, the same antioxidants that can be used to prevent oxidation deterioration in food can also be used in some cases in functional foods design.

As a following step, and looking also for increasing the applicability of wheat bran oil, its formulation in oil-in-water nanoemulsions has been considered. Different targets have been searched with this formulation: on the one hand, wheat bran extracts can be used in water systems, which due to their lipophilic character is not possible without using a formulation strategy and also, nanoemulsification has been described as a suitable way for increasing the stability and bioactivity of active compounds. These formulated systems should be designed taking into account physicochemical, sensory and biological characteristics and therefore, the optimization of the nanoemulsion composition and emulsification process and the study of the emulsions stability was carried out.

In the final part of this work, the suitability of the wheat bran nanoemulsions to inhibit enzymes, which are related to melanosis and subsequent loss of quality and acceptability of food products, has been evaluated. As it has been previously mentioned, there is growing interest in replacing synthetic additives, such as sulfites, and that is the reason why the food industry is continuously looking for natural effective ones. Therefore, the effectiveness of wheat bran nanoemulsions to inhibit some enzymes has been studied, specifically, inhibition of a commercial mushroom tyrosinase and a directly extracted polyphenoloxidase from prawn has been evaluated and related to the wheat bran oil composition and the antioxidant activity of its nanoemulsions.

RESUMEN

La adición de antioxidantes es la estrategia más utilizada en la industria alimentaria para preservar la calidad de los alimentos, retardando su deterioro, rancidez o decoloración debida a la oxidación. Antioxidantes fenólicos sintéticos como el butilhidroxianisol (BHA), butilhidroxitolueno (BHT) y el galato de propilo son inhibidores efectivos de la oxidación y por tanto, comúnmente utilizados como aditivos. Otros antioxidantes ampliamente utilizados son el ácido ascórbico y los tocoferoles, tanto naturales como sintéticos, y los sulfitos. Entre las funciones de los sulfitos se incluyen la inhibición del pardeamiento, enzimático y no enzimático, así como el control del crecimiento microbiano en distintos tipos de alimentos. La principal desventaja que presentan estos aditivos se debe a su relación con reacciones alérgicas en los consumidores, lo que ha dado lugar a una regulación cada vez más estricta para su aplicación en productos alimentarios. La creciente demanda por parte de los consumidores de productos naturales y el efecto negativo para la salud de algunos aditivos está promoviendo en los últimos años la búsqueda de antioxidantes naturales alternativos que puedan proteger los alimentos de la oxidación y el pardeamiento y su consecuente pérdida de calidad.

En los últimos años, diferentes materias primas naturales como especias y hierbas, té y semilla de uva entre otros, han sido utilizadas como fuentes de antioxidantes. En este sentido, el salvado de trigo surge como una valiosa fuente de antioxidantes debido a su contenido en compuestos bioactivos. Además, el salvado de trigo es un producto fácilmente disponible debido a que se obtiene como un subproducto durante el proceso de molienda del trigo y es principalmente destinado a alimentación animal.

El presente trabajo de investigación se ha centrado en la extracción de compuestos bioactivos del salvado de trigo utilizando una tecnología limpia, la extracción con fluidos supercríticos, que puede contribuir tanto a la búsqueda de nuevos antioxidantes naturales con potencial aplicación en la industria alimentaria como a la valorización de un subproducto de la industria alimentaria.

Los métodos más comúnmente utilizados para la extracción de compuestos bioactivos son la extracción con disolventes y la destilación por arrastre de vapor y habitualmente implican la utilización de grandes cantidades de disolventes, largos tiempos de operación y/o altas temperaturas. Para superar estas desventajas y para incrementar los rendimientos de extracción y la calidad de los extractos obtenidos, en las últimas décadas

se han desarrollado diferentes técnicas. La alta versatilidad del proceso de extracción con fluidos supercríticos, que no utiliza disolventes orgánicos, hace que se haya convertido en una tecnología apropiada para la extracción de productos de alto valor añadido de diferentes matrices alimentarias. En el caso concreto de este trabajo, del salvado de trigo. El estudio de la extracción con fluidos supercríticos aplicada al salvado de trigo ha sido el principal objetivo de la primera parte de este trabajo, donde se ha llevado a cabo la evaluación del efecto de diferentes parámetros que influyen en el proceso. Estos parámetros incluyen tanto las condiciones de extracción y el modo de operación como otros parámetros relacionados con las características de la materia prima.

Además del estudio del proceso de extracción, ha sido también objetivo de este trabajo la evaluación de la composición y calidad del aceite extraído. El salvado de trigo es fuente de diversos componentes muchos de los cuales han sido reconocidos como antioxidantes efectivos. La caracterización del aceite extraído y la evaluación de su actividad antioxidante han sido consideradas fundamentales durante todo el desarrollo de este trabajo con el objetivo de determinar el potencial uso y aplicaciones en la industria alimentaria del aceite de salvado de trigo obtenido, dado que los mismos antioxidantes que pueden ser utilizados para prevenir el deterioro de los productos debido a la oxidación, pueden ser utilizados en muchos casos como ingredientes en el diseño de alimentos funcionales.

Como etapa siguiente, y buscando aumentar las posibles aplicaciones del aceite de salvado de trigo, se consideró su formulación en nanoemulsiones de aceite en agua. Con esta formulación se ha buscado por un lado permitir la incorporación del aceite de salvado de trigo en sistemas acuosos, lo cual no es posible sin su formulación debido al carácter lipofílico del aceite, y por otro, aprovechar las ventajas de la nanoemulsificación, que se ha descrito como una técnica adecuada para incrementar la estabilidad y bioactividad de los compuestos activos. Estas formulaciones deben diseñarse teniendo en cuenta las características fisicoquímicas, sensoriales y biológicas de los productos a formular y por ello, se ha llevado a cabo la optimización de la composición de la nanoemulsion y el proceso de emulsificación así como el estudio de la estabilidad de las emulsiones.

En la parte final de este trabajo se ha evaluado la eficacia de las nanoemulsiones de aceite de salvado de trigo para inhibir enzimas relacionadas con la melanosis y con la consecuente pérdida de calidad y aceptabilidad de distintos productos alimentarios. Como se ha descrito anteriormente, cada vez hay interés mayor por sustituir los aditivos sintéticos, como los sulfitos, por compuestos naturales, por lo que la industria alimentaria

está continuamente buscando antioxidantes naturales efectivos. Por ello, se ha evaluado la capacidad de las nanoemulsiones de aceite de salvado de trigo para inhibir algunas enzimas como son, la tirosinasa comercial de champiñón y la polifenoloxidasa extraída de langostino y se ha relacionado esta capacidad con la composición del aceite de salvado de trigo y la actividad antioxidante de sus nanoemulsiones.

Introduction

ABSTRACT

A general introduction to this work is presented in this chapter. The characteristics and composition of the raw material used, wheat bran, are discussed in the first place. The interest of the different wheat bran phytochemicals and their biological activities is then presented. A brief approach to the supercritical fluid extraction process which was applied for obtaining the bioactive compounds of the wheat bran is also shown. Furthermore, an introduction to the formulation process of bioactive compounds for their application into food matrices is presented. Finally, a description of the enzymatic browning occurring in food products is included.

An extensive introduction is showed with more detail in the introduction section of each chapter.

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1. WHEAT BRAN

Wheat belongs to the genus *Triticum* of the grass family Graminae, being the hexaploid type the major class of wheat, known as "common" wheat (Dendy & Dobraszczyk, 2001). The worldwide production of wheat was estimated to be of 690 million tons during 2013 (www.fao.org/docrep/017/al998s/al998s.pdf) and approximately 72% are used as food and 16% as a source of animal feed (Dendy & Dobraszczyk, 2001).

The wheat grain can be divided into three distinct morphological parts (Figure I.1): the endosperm which makes up most of the grain; the bran layer, which surrounds the grain; and the germ, which includes the embryo and the scutellum. Wheat bran, which is supposed to be 14-19% of wheat grain, is obtained as a by-product during the wheat milling process when the endosperm is separated to produce wheat flour (Dendy & Dobraszczyk, 2001). The raw material used in this work was provided by Harinas y Sémolas del Noroeste S.A (HASENOSA) and it was bran from three *Triticum aestivum* L. varieties and their mix (Soissons, Altria and Nogal), being Soissons the most abundant (around 80%).

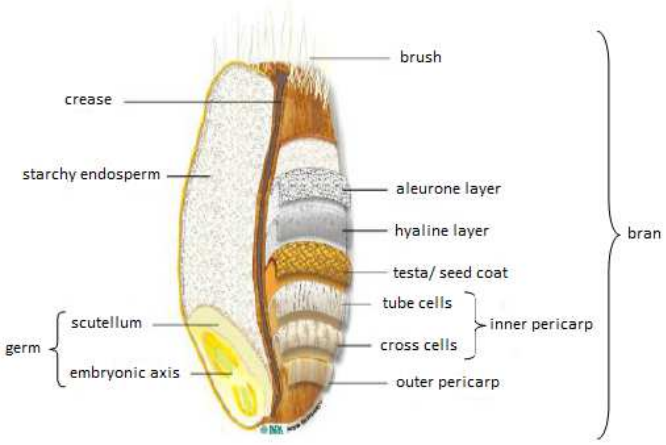


Figure I. 1. Histological composition of wheat bran. Taked from (Javed et al., 2012)

Wheat bran is mainly composed of non-starchy carbohydrates and proteins, with a low amount of fat (Table I.1). The lipid fraction is mainly formed by unsaturated fatty acids, linoleic and oleic acids (Burkwall & Glass, 1965), what make this fraction interesting from a nutritional point of view due to the low amount of saturated fatty acids but with the drawback of being more susceptible to the oxidation.

Wheat bran is also a valuable source of biologically active compounds such as dietary fiber, sterols, tocopherols, tocotrienols, alkylresorcinols, phenolic acids, and vitamins most of them of interest in nutrition, pharmacy and cosmetics (Bartlomiej et al., 2012). Then, a short description of the bioactive compounds object of this work and their biological activities is presented.

Table I.1. Approximate composition of wheat bran*

Compound	%
Moisture	13.2
Protein	14.4
Fat	4.7
Ash	6.3
Carbohydrate	61.4

*(Dendy & Dobraszczyk, 2001)

1.1. Alkylresorcinols

Alkylresorcinols (AR) are a group of phenolic lipids characterized by a benzene ring with two hydroxyl groups at position 1 and 3 (hydrophilic head) and a non-isoprenoid alkyl chain attached at position 5 (hydrophobic tail) (Table I.2). AR homologues are classified according to the length of the alkyl chain (11-29 carbon atoms) and the degree of unsaturation (0-4 double bonds). Among commonly consumed foods, AR are mainly found in the outer parts of wheat and rye grains. Due to the presence in these outer parts of the kernel, they are found in high amounts only in whole grain and bran products and in low amounts in refined flour products (Landberg et al., 2013). The amphiphilic character, consequence of the hydrophilic and hydrophobic regions previously described, is an important aspect related with the analysis, absorption, metabolism and potential bioactivity of AR (Sampietro et al., 2013). Some of the bioactivities reported for the AR present in the cereal bran are as antioxidant, antimicrobial and anticancer compounds and enzyme inhibitors among other activities (Table I.3).

Table I.2. Chemical structure of some bioactive phytochemicals found in wheat bran

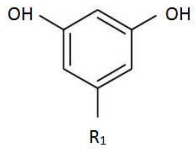
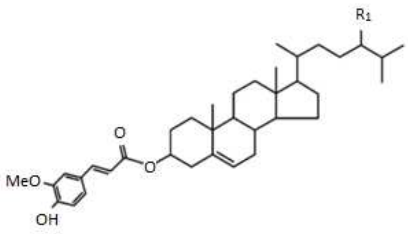
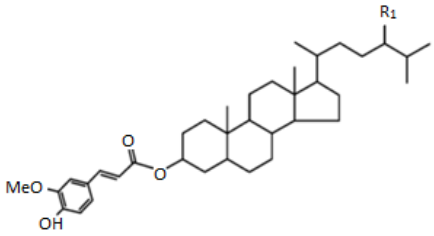
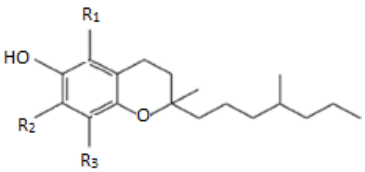
Compound		R_1	R_2	R_3
Alkylresorcinols				
C15-AR		$C_{15}H_{31}$	-	-
C17-AR		$C_{17}H_{35}$	-	-
C19-AR		$C_{19}H_{39}$	-	-
C21-AR		$C_{21}H_{43}$	-	-
C23-AR		$C_{23}H_{47}$	-	-
C25-AR		$C_{25}H_{51}$	-	-
Steryl ferulates				
Campesteryl ferulate		H	-	-
Sitosteryl ferulate		CH_3	-	-
Campestanyl ferulate		H	-	-
Sitostanyl ferulate		CH_3	-	-
Tocopherols				
α-tocopherol		CH_3	CH_3	CH_3
β-tocopherol		CH_3	H	CH_3
γ-tocopherol		H	CH_3	CH_3
δ-tocopherol		H	H	CH_3

Table I.3. Activities and potential uses of some active compounds found in wheat bran

Bioactive compounds	Bioactivities	Potential use	Reference
Alkylresorcinols	In vitro antioxidant activity	Antioxidants in food systems	Korycinska et al. (2009)
	C15:0 isolated from *Anacardium occidentale* fruits slightly inhibits mushroom tyrosinase	Antibrowning agent	Kubo et al. (1994)
	Inhibition of disease symptoms on fruits inoculate with *Penicillium expansum*	Antimicrobial agents	Dey et al. (2012)
	Inhibition of glycerol-3-phosphate dehydrogenase and triglyceride accumulation prevention in 3T3-L1 cells	Prevention of obesity	Rejman and Kozubek (2003)
	Inhibition of catecholamine-stimulated lipolysis in adipocytes and hormone-sensitive lipase activity	Protective effect in diabetes risk	Andersson et al. (2011)
	Inhibition of colon cancer cell growth	Anticancer agents	Zhu et al. (2011)
Steryl ferulates	Antioxidant activity in bulk and emulsified methyl linoleate	Antioxidants in lipid systems	Nyström et al. (2005)
	Inhibitory activity in Epstein-barr virus activity	Antitumor promoters	Iwatsuki et al. (2003)
Tocopherols	Reduction of lipid oxidation in salmon package with tocopherol active films	Prevention of lipid oxidation in food systems	Barbosa-Pereira et al. (2013)
	γ-T-rich mixture of tocopherols is beneficial for maintaining vascular endothelial function in healthy young men	Cardiovascular disease risk mitigation	Mah et al. (2013)
	α- and γ-T and their metabolites inhibit prostatic cancer cells	Prevention and therapy of prostate cancer	Galli et al. (2004)

1.2. Steryl ferulates

Ferulic acid esters of plant sterols and their saturated form, stanols (Table I.2), are mostly found in the bran fraction of wheat grain, in contrast to sterols which are found in both germ and bran fractions. Approximately 17-24% of the phytosterols in the bran are bound to ferulic acid (Nurmi et al., 2012).

Steryl ferulates are considered to be potential antioxidants because of the hydrogen-donating ability of the phenolic group of ferulic acid (Nyström et al., 2005). Ferulic acid, like various other common antioxidants used in food applications (BHA, BHT, and TBHQ), is a small and polar compound with only a limited solubility in oils. Once esterified to a nonpolar compound like phytosterol, the polarity of the molecule is decreased and solubility in oils is improved (Nyström et al., 2007). Bioactivity of steryl ferulates from rice bran, known as γ-oryzanol, has been widely reported while bioactivity of wheat bran steryl ferulates has been less studied (Table I.3).

1.3. Tocopherols

Tocopherols are derivates of 2-methyl-6-chromanol onto which a saturated 16-carbon isoprenoid chain is attached at C2. Each tocopherol homologue (α-, β-, γ- and δ) differs in the number and positions of the methyl groups (Table I.2) (Bramley et al., 2000). The main sources of tocopherols in the human diet are vegetable oils and also cereal grains, being wheat and rye the richest sources (Bartlomiej et al., 2012). Since tocopherols are found mainly in the outer layers of the grain, the tocopherol content is enriched in certain fractions, such as bran and germ, during milling (Andersson et al., 2014).

The interest in tocopherols resides in its vitamin E and antioxidant activities which vary among the different homologues. Tocopherols are usually employed lipophilic antioxidants in food systems and they have also shown a potentially beneficial effect in the prevention of cardiovascular diseases, cancer or neurological disorders among others (Table I.3) (Bramley et al., 2000; Seppanen et al., 2010).

Tocopherols isolated from natural sources are more biologically active than their synthetic counterparts. The biological activity is defined in terms of α-tocopherol equivalents (α-TE). Natural α-tocopherol has an activity of 1 mg α-TE/mg compound and the activities for natural β-, γ- and δ-tocopherol are 0.5, 0.1 and 0.03 mg α-TE/mg compound, respectively (Bramley et al., 2000; Gunstone, 2013).

Regarding the antioxidant activity, different methodologies have been applied to investigate the antioxidant activities of tocopherols in oils and fats showing α-tocopherol better antioxidant activity than γ-tocopherol at lower hydroperoxides concentration while γ-tocopherol was found to be a more active antioxidant at higher concentrations (Seppanen et al., 2010).

2. SUPERCRITICAL FLUID EXTRACTION

A wide variety of process related to polymer science (foaming, additive blending), catalysis (hydrogenation), and biotechnology (enzymatic reactions) have been developed using supercritical fluid technology but, the main application of this technology in the food industry is supercritical fluid extraction (SFE) (Beckman, 2012).

It is called SFE to the process in which a supercritical fluid is used as extracting solvent; that is, at a pressure and temperature above its critical point. Under these conditions, the supercritical fluid shows a density similar to a liquid and a viscosity similar to a gas being its diffusivity intermediate between the two states so they can diffuse easily through solid materials giving higher extraction yields than conventional solvent extraction.

Several extracting compounds can be used in supercritical fluid extraction, but CO_2 is by far the most used. It shows mild critical conditions (T_c= 304.15 K and P_c= 7.38 MPa), it is not flammable, non-toxic, gas at ambient temperature, naturally abundant and hence inexpensive (Beckman, 2012). Supercritical CO_2 (SC-CO_2) is a good solvent for lipophilic (non-polar) compounds, whereas, it has a low affinity with polar compounds. This fact can be overcome adding a liquid cosolvent to increase its solvent power towards polar molecules but this strategy has the drawback that, a larger solvent power could also mean lower process selectivity and since, as a rule, the cosolvent is liquid at atmospheric pressure, it will be collected in the separator together with the extracted compounds. Subsequent processing for solvent elimination is required; therefore, one of the advantages of the SFE, which is solventless operation, is lost (Reverchon & Marco, 2006).

Gas extraction from solids consists of two process steps, the extraction and the separation of the extract from the solvent. In the extraction, the supercritical solvent flows through a fixed bed of solid particles and dissolves the extractable components of the solid. The loaded solvent is removed from the extractor and fed to the precipitator (Brunner, 1994).

The extraction of soluble compounds from solid plant material proceeds in several parallel and consecutive steps (Brunner, 1994):

1) The plant matrix absorbs the supercritical solvent
2) The extract compounds are dissolved by the solvent
3) The dissolved compounds are transported to the outer surface of the solid. Diffusion is the most important transport mechanism.
4) The dissolved compounds pass through the outer surface. A phase change may occur at that place.

The compounds are transported from the surface layer into the bulk of the supercritical solvent and are subsequently removed with the solvent from the bulk of solid material.

The basic extraction scheme consists of a pump, an extraction vessel charged with the raw material to be extracted and a depressurization valve at the exit of the extractor, which flows the supercritical fluid (SCF) into the separator where due to the lower pressure, the extracts are released from the gaseous medium and collected. Additional separators could be added to obtain different composition fractions by setting different temperatures and pressures in each separator. Other operation modes include cosolvents addition or multistage extraction. In the last one, the extraction process takes place in several steps in which pressure and/or temperature are varied and therefore the solvent power of SC-CO_2 in each step is changed. This strategy can be used when the extraction of several compound families from the same matrix is required and they show different solubilities in SC-CO_2 (Reverchon & Marco, 2006).

Pressure and temperature, are the main parameters that influence the extraction process. The density, diffusivity, viscosity, and dielectric constant of the SCF can be controlled by varying the operating conditions of pressure and temperature, or both in combination. Thus, the separation process can be affected by simply changing the operating pressure and temperature to alter the solvating power of the solvent (Shi et al., 2012).

In general, the solvent capacity increases with pressure at a constant temperature and it is reduced with an increase of temperature, at a fixed pressure. However, the vapor pressure of the compounds to be extracted increases with temperature and therefore, a higher temperature often causes a higher extraction rate. Frequently, the solvent power is described in terms of the SC-CO_2 density which is connected to both temperature and pressure conditions (Reverchon & Marco, 2006).

Other important process parameters in SFE are CO_2 flow rate, particle size of the matrix and extraction time. They are connected to the thermodynamics (solubility) and the kinetics of the extraction process in the specific raw matter (mass transfer resistances).

The process velocity is determined by the mechanism which controls the process that is, the slowest one. CO_2 flow rate is a relevant parameter if the process is controlled by an external mass transfer resistance or by equilibrium: the amount of supercritical solvent feed to the extraction vessel, in this case, determines the extraction rate. Particle size plays a determining role in extraction processes controlled by internal mass transfer resistances, since a smaller mean particle size reduces the length of diffusion of the solvent. However, if particles are too small, problems of channelling inside the extraction bed can take place: part of the solvent flows through channels formed inside the extraction bed and does not contact the material to be extracted thus causing a loss of efficiency and yield of the process. Process duration is interconnected with CO_2 flow rate and particle size and has to be properly selected to maximize the yield of the extraction process (Reverchon & Marco, 2006).

2.1. SFE of bioactive compounds

A wide range of high value added compounds has been extracted from natural sources using supercritical CO_2 in the last decades due to their potential application into the food, chemical and pharmaceutical industries (Azmir et al., 2013; Reverchon & Marco, 2006).

Bioactive compounds from plant materials can be extracted by various classical extraction techniques that are usually based on the extracting power of different solvents and the application of heat and/or mixing such as solvent extraction, maceration and hydrodistillation (Azmir et al., 2013). These conventional extraction techniques usually exhibit some disadvantages such as long extraction times, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds. To overcome these limitations some non-conventional extraction techniques in which SFE is included have been developed in the last years for the extraction of bioactive compounds from plant sources (Azmir et al., 2013).

SC-CO_2 extraction has been applied to different vegetal sources in order to obtain extracts rich in bioactive compounds that have shown for example antioxidant and antimicrobial activities such as: essential oils (Danh et al., 2012; Fornari et al., 2012), tocopherols (Kraujalis & Venskutonis, 2013; Nyam et al., 2010), γ-oryzanol (Jesus et al., 2010; Tomita et al., 2014) and sterols (Gil-Ramírez et al., 2013).

Most SC-CO_2 applications extract natural products or bioactive compounds at pressures ranging between 20 and 50 MPa, and at temperatures between 40 °C and 80 °C. Because of the poor solubility of some bioactive substances in SC-CO_2 , food and/or pharmaceutical

grade modifiers, ranging from 3% to 20%, are sometimes added to help the extraction of more polar compounds from plant materials (Shi et al., 2012).

The application of SFE in cereal bran reported in the literature is discussed in the introduction sections corresponding to Chapters 1 and 2.

3. FORMULATION OF BIOACTIVE COMPOUNDS FOR ITS APPLICATION INTO FOOD MATRICES

There are a number of applications where lipophilic active components (such as bioactive lipids, flavours, antimicrobials, antioxidants, nutraceuticals, and drugs) need to be incorporated into aqueous media. One of the most convenient means of achieving this aim is to encapsulate the lipophilic active components within colloidal-based delivery systems, such as nanoemulsions (Ziani et al., 2012). Besides allowing the incorporation of active compounds in aqueous systems, encapsulation is also used to increase its bioavailability, to control the rate or location of its release or to protect them from chemical degradation (McClements, 2011).

Basically, an emulsion consists of at least two immiscible liquids, usually oil and water, with one of the liquids being dispersed as small spherical droplets in the other. A system that consists of oil droplets dispersed in an aqueous phase is called and oil-in-water (O/W) emulsion, whereas a system that consists of oil droplets dispersed on an oil phase is called water-in-oil (W/O) emulsion (Figure I.2). Furthermore, various types of multiple emulsions can be developed such as oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) emulsions (Fang & Bhandari, 2010). It is possible to form emulsions that are kinetically stable for a reasonable period of time by including substances known as stabilizers, e.g., surfactants, which are surface-active molecules that adsorb to the surface of freshly formed droplets during homogenization, forming a protective layer that prevents the droplets from aggregating (Piorkowski & McClements).

A nanoemulsion can be considered to be a conventional emulsion that contains very small droplets (10-100 nm). The relatively small droplet size compared to the wavelength of light means that they tend to be transparent or only slightly turbid so they can be applied in products that need to be optically transparent. In addition, the small droplet size means that they have much better stability to gravitational separation and aggregation than conventional emulsions. Nanoemulsions have also shown to improve the bioavailability of various lipophilic nutraceuticals and pharmaceuticals, increasing this bioavailability when the droplet size is decreased (McClements, 2011).

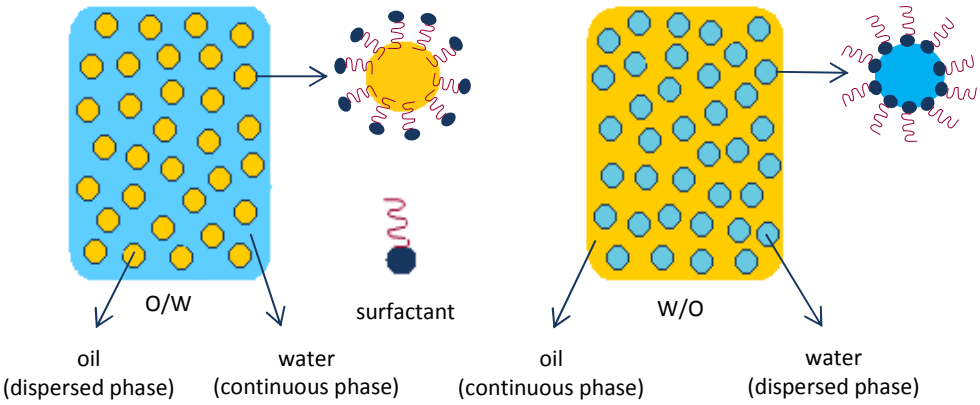


Figure I.2 Schematic representation of oil-in-water (O/W) and water-in-oil (W/O) emulsions

Some active components that have been formulated in O/W nanoemulsions for their incorporation into food, cosmetic or pharmaceutical systems are for example β-carotene (Yuan et al., 2008), vitamin E (Mayer et al.) and coenzyme Q_{10} (Cho et al., 2014), which are previously dissolved in different oily phases such as medium chain triglycerides or sunflower oil before their emulsification by high or low intensity emulsification processes.

More details about nanoemulsion composition and production are discussed in the introduction of Chapter 4.

4. ENZYMATIC BROWNING IN FOOD PRODUCTS

The colour change which occurs in plant and animal material after cell disruption and results in the formation of brown or sometimes yellow, black, or pink pigments is mainly due to enzymatic processes. Cell disruption may arise from mechanical injury, temperature changes or cell death and results in the decompartmentation of phenolic substrates and enzymes and then the oxidative production of colored pigments (Nicolas et al., 2003).

Enzymatic browning is catalyzed by a group of enzymes known as polyphenol oxidases (PPO), which accomplish the hydroxylation of monophenols into *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols into *o*-quinones (diphenolase activity) with oxygen as the primary oxidant (Figure I.3). This is followed by non-enzymatic

polymerization of the quinones to form high molecular weight compounds or brown pigments (melanins) (Yoruk & Marshall, 2003).

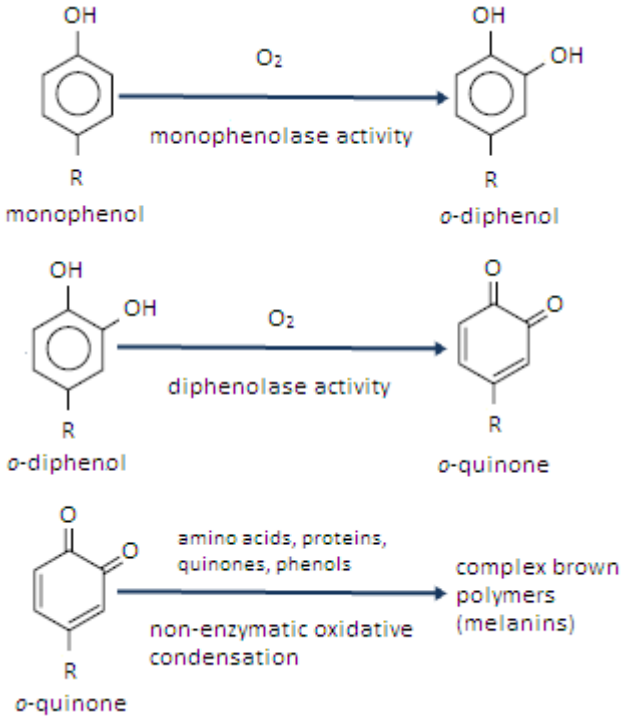


Figure I.2. Schematic representation of enzymatic and subsequent non-enzymatic condensation processes for browning. Adapted from (Yoruk & Marshall, 2003)

Enzymatic browning results in a quality loss in many food products such as fruits and vegetables during their processing and storage. Also, animal products such as crustaceans are affected by enzymatic browning which leads to the formation of black spots (melanosis) in the product. Although the pigmentation seems to be harmless for the consumer, it drastically reduces the market value of the products causing considerable financial losses (Montero et al., 2001).

Traditionally sulphites have been used to inhibit the enzymatic oxidation and browning in 'fresh-cut' and processed fruits and seafood. Nevertheless, the increase in regulatory attention and consumer awareness of some risks associated with sulphites has drawn some attention to other anti-oxidation and anti-browning alternatives. As in the case for

other food additives, the food industry is particularly interested in the use of browning inhibitors from natural sources rather than synthetic (Valverde, 2013).

Inhibitory PPO activity of different natural and synthetic compounds is described with more detail in Chapter 5.

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Objectives

OBJECTIVES

The main objective of this work was the upgrading of a by-product that is obtained during the wheat milling process, thus by-product is wheat bran. The strategy proposed for achieving this objective was the extraction of a high-value added oil which could be of interest for food applications due to its content in bioactive compounds and antioxidant activity. The extraction process applied was supercritical fluid extraction with CO_2, a generally recognized as safe (GRAS) technology.

The specific objectives of the present work are:

1. Optimization of the supercritical fluid extraction of wheat bran

The optimization of the extraction process of wheat bran oil with supercritical CO_2 was carried out using both, a laboratory and pilot supercritical extraction plants. The influence of different process parameters has been studied in order to obtain the extraction kinetics and to model the process. Also, a comparison with organic solvent extraction was assessed. Besides the extraction yield, other parameters such as the alkylresorcinol and polyphenol content of the oil and its antioxidant activity have been taken into account in the study of the extraction process.

2. Characterization and quality evaluation of the extracted oil

A wide characterization of the wheat bran oil was carried out in order to determine its bioactive compounds composition. Quality and antioxidant activity was evaluated and related to the oil composition and its evolution during storage.

3. Formulation of the oil for its application into food matrices

Nanoemulsification was applied as a formulation strategy to increase the bioavailability of the wheat bran oil on water systems. The composition of the emulsion and the emulsification process was optimized and a wide characterization of the optimal nanoemulsion was performed.

4. Evaluation of polyphenoloxidase inhibition potential of wheat bran oil nanoemulsions

The potential of wheat bran oil as antibrowning agent was evaluated. The inhibition capacity of wheat bran nanoemulsions on mushroom tyrosinase and prawn polyphenoloxidase was assessed.

The specific objectives and main tasks together with their link to the different chapters are shown in more detail in Table O.1.

Table O.1. Specific objectives and main tasks of the present work

Specific objective	Chapter	Main tasks
Optimization of the supercritical fluid extraction of wheat bran oil	Chapter 1	• Comparison of conventional organic solvent and supercritical fluid extractions • Study of the influence of process parameters (particle size, static extraction pretreatment and temperature) on extraction yield, composition and antioxidant activity • Modeling of SFE process
	Chapter 2	• Study of the influence of process parameters (wheat bran moisture, pressure and temperature) on extraction yield, composition and antioxidant activity • Fractionation of wheat bran oil by using two-stage extraction
Characterization and quality evaluation of the extracted oil	Chapter 3	• Determination of bioactive compounds content • Evaluation of oil quality • Determination of oil antioxidant activity • Evolution study of wheat bran oil during storage
Formulation of the oil for its application into food matrices	Chapter 4	• Optimization of nanoemulsion composition and emulsification process • Determination of optimal nanoemulsion characteristics and stability
Evaluation of polyphenoloxidase inhibition potential of wheat bran oil nanoemulsions	Chapter 5	• Determination of wheat bran oil nanoemulsions inhibition capacity on mushroom tyrosinase • Determination of wheat bran oil nanoemulsions inhibition capacity on polyphenoloxidase extracted from prawn (*Marsupenaeus japonicus*)

Chapter 1

Supercritical fluid extraction of wheat bran oil: comparison with conventional solvent extraction and extraction kinetics

Rebolleda, S., Beltrán, S., Sanz, M. T., González-Sanjosé, M. L., & Solaesa, Á. G. (2013). Extraction of alkylresorcinols from wheat bran with supercritical CO_2. *Journal of Food Engineering*, 119 (4), 814-821.

ABSTRACT

The supercritical fluid extraction of wheat bran alkylresorcinols has been studied. Extractions were carried out at 40.0 MPa. The effect of particle size, static extraction pretreatment with supercritical CO_2 and extraction temperature on the extraction kinetics was investigated. The extraction yield increased as the particle size decreased and with temperature. Extraction curves present a faster and linear initial extraction period followed by a slower extraction period. Based on these results the approximate mathematical model of Sovová was successfully applied to describe the extraction curves. The total content of alkylresorcinols was determined and compared with the alkylresorcinol content obtained by conventional organic solvent extraction. Due to the amphiphilic nature of these resorcinolic lipids, the extraction yield was higher for polar organic solvents than for SC-CO_2. Characterization of supercritical extracts was also performed by determining the fatty acid composition and antioxidant activity.

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1. INTRODUCTION

Alkylresorcinols (AR) are amphiphilic 1,3-dihydroxybenzene derivatives with a long odd-numbered alkyl chain (15-25 carbon atoms) at position 5 of the benzene ring (Ross et al., 2003). AR are an important class of secondary metabolites that occur in bacteria, algae, mosses, fungi, animals and higher plants (Athukorala et al., 2010). AR are mainly found in the bran fraction of cereal grains and, consequently, are largely missing in refined cereal flour and conventional cereal products. These compounds represent about 85% of total cereal grain resorcinolic lipids (Francisco et al., 2005a). Among cereal species, the bran fraction of wheat and rye presents high levels of AR (32-143 and 36-320 mg/100 g dry matter, respectively). AR have been reviewed as protective antioxidants in biological membranes and as having stimulant or inhibitory effects on some metabolic enzymes (Bondia-Pons et al., 2009).

Traditionally, different organic solvents have been used to extract AR from the bran fraction of the Gramineae family (Agil et al., 2012; Mattila et al., 2005; Zarnowski & Suzuki, 2004; Zhou & Yu, 2004). However, organic solvent extraction usually requires laborious purification of the extracts.

Francisco et al. (2005a, 2005b) reported, for the first time, the use of supercritical carbon dioxide (SC-CO_2) technology for AR extraction from cereal milling by-products, specifically from rye bran. However, in these studies none of the AR homologues were detected in the extract when pure CO_2 was used within the imposed operative conditions (35 MPa; 55 °C-70 °C). Therefore, they proposed the use of ethanol as a polar cosolvent to improve the extractability of AR, upon bran delipidation with pure SC-CO_2. Previous to the extraction with cosolvent a pre-extraction with pure SC-CO_2 was performed to remove a fraction that did not contain AR. Atukorala et al. (2010) also reported AR extraction from triticale bran by a two-step SC-CO_2 extraction since at the operating conditions (35 MPa and 70 °C) trace amounts of AR were detected when using pure SC-CO_2. Dey and Mikhailopulo (2009) proposed a two-step extraction process to pre-purify AR during the extraction process from rye bran. In the first step, low concentrations of ethanol cosolvent were used and higher ethanol cosolvent concentrations were used in the second step. However, higher amounts of AR were removed during the first extraction step. At 70 °C and 25 MPa, by using 0.06% of ethanol as cosolvent in the first step, 1054 µg AR/g dry mater was obtained; while at lower temperatures (45 °C), by using 10% of ethanol in the second step, 381 µg AR/g dry matter was obtained. Based on these results and on

previous results obtained in our laboratory, in this work the extraction capability of pure SC-CO_2 on AR from wheat bran has been studied. Solvent power of SC-CO_2 has been improved by working at higher pressures than in previous works related with AR extraction by using SC-CO_2.

The objective of this chapter was the study of the extraction capability of pure SC-CO_2 on AR from wheat bran. Extraction curves at different operating parameters, such as particle size, static extraction pretreatment with SC-CO_2, and extraction temperature, have been obtained. The Sovova's mathematical model (Sovová, 2005) was used to describe the extraction kinetics. This way, parameters that could help to a better understanding of the extraction process have been estimated. Characterization and comparison of wheat bran extracts obtained by SC-CO_2 and conventional organic solvent extraction has been performed in terms of their AR and fatty acid profile and antioxidant activity evaluated by different methods.

2. MATERIALS AND METHODS

2.1. Raw material

The wheat bran (*Triticum aestivum*, L.) was kindly supplied by HASENOSA (Spain). The particle size distribution of wheat bran was determined by using a vibratory sieve shaker (Cisa model RP.09) and it is reported in Table 1.1. The moisture content of the wheat bran, determined by drying in an oven at 105 °C to constant weight, was found to be around 13 ± 1% (w/w).

Table 1.1. Particle size distribution of wheat bran

Particle size, p	Mass percentage (%)
p < 0.5 mm	14.01
0.5 mm < p < 2 mm	81.24
p > 2 mm	4.75

2.2. Conventional solvent extraction

The conventional solvent extraction of wheat bran was carried out by using three different solvents, acetone (Merck, 99.9%), ethanol (Merck, ≥99.9%) and petroleum ether (Merck, analytical reagent). Acetone is used in most extraction procedures for AR isolation (Zarnowski & Suzuki, 2004), ethanol is of interest since it is often used as cosolvent to modify the solvent power of supercritical CO_2 and petroleum ether was considered due to its similar polarity to CO_2.

Conventional solvent extraction experiments are summarized in Table 1.2. Two organic extraction methods were used: continuous shaking at room temperature and Soxhlet extraction. In the first case (R1 and R2) 4-6 grams of raw material were extracted with 50 mL of solvent (acetone or ethanol) in a glass flask during 24 h. After the extraction time, the extracts were filtered through paper filters and evaporated under vacuum using a rotary evaporator (Heibolph VV2000). In R3 and R4, Soxhlet extractions were done in a Buchi equipment (B-811 model) using 25 extraction cycles to put the sample (1 g) in contact with the solvent (acetone or petroleum ether) at its boiling temperature, followed by rinsing and drying steps. Extraction experiments were replicated twice.

2.3. Supercritical fluid extraction equipment and procedure

The extraction experiments were carried out in a laboratory SFE-plant (Appendix A). In a SFE experiment, 6-8 grams of wheat bran were loaded in the extractor (40 mL capacity). Two syringe pumps (ISCO 260 DM), that work alternatively, provide an uninterrupted flow of CO_2 (Carburos metálicos, liquid CO_2 ≥ 99.9 %) compressed up to the desired operating pressure, 40.0 MPa. The pressurized solvent was pre-heated up to the desired extraction temperature before entering the extractor. The extractor was held in an oven whose temperature is controlled within an accuracy of ± 0.5 °C. The carbon dioxide flow was set to 1.5 ± 0.3 g/min. Depressurized CO_2 was quantified with a totalizer flow meter. Extraction yield was determined gravimetrically by measuring the extract weight at different time intervals. Extraction parameters evaluated to study the extraction of wheat bran oil were: particle size, static extraction pretreatment raw material-SC-CO_2 at the extraction pressure and extraction temperature. A total of ten experiments were carried out under different extraction conditions (Table 1.3). Runs 5 to 8 were performed to evaluate the influence of the particle size on the extraction yield. Runs 8 to 10 were carried out to study the influence of static extraction pretreatment with SC-CO_2. Runs 11

to 14 and run 9 were carried out to determine the effect of extraction temperature. Most of the extractions were replicated twice.

2.4. Analytical methods

A full description of the analytical methods used in this chapter can be found in the Appendix B.

2.4.1. Chemicals and materials

Fast Blue RR salt, olivetol, AR standards (C15, C17, C19 and C25), fatty acid methyl esters Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl) and TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine), were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). K_2CO_3, $FeCl_3$ and $FeSO_4$ were obtained from Panreac (Barcelona, Spain) and methyl tricosanoate by Larodan (Malmö, Sweeden).

2.4.2. Determination of total AR

The total AR content in the extracted material was determined by a colorimetric method based on the use of Fast Blue RR salt (Sampietro et al., 2009). A stock solution of 0.05% Fast Blue RR reagent was used to prepare a working solution by mixing 1 part of stock reagent with 4 parts of methanol. Aliquots (20 µL) of methanol solutions of wheat bran extracts (5 mg/mL) were placed in assay tubes and made up to 200 µL with methanol. Then, 2 mL of the working solution and 10 µL of a 10% K_2CO_3 solution were added to each tube. Absorbance of the reaction mixture was measured at 480 nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan) after 20 min. Olivetol (5-pentylresorcinol) was used as internal standard.

2.4.3. Determination of AR profile

Alkylresorcinols were determined according to a modification of the method proposed by Knödler et al. (2008) using an Agilent HPLC (series 1100) equipped with a diodo array detector and a mass spectrometry detector with an APcI source. The column used was Kromasil C18-5 250 x 4.6 mm that operated at 25 ºC. The mobile phase was methanol (A) and water (B) and the following gradient was used: 2% B to 0% B in 10 min. The total run time was 100 min. The injection volume was 100 µL. All AR were monitored at 280 nm at a flow rate of 0.6 mL/min.

Positive-ion mass spectra of the column eluate compounds were recorded in the range m/z 100–500. Nitrogen was used both as the drying gas at a flow rate of 10 L/min and as

the nebulizing gas at a pressure of 380 Pa. The nebulizer temperature was set at 350 °C and a potential of 4000 V was used on the capillary.

Individual compounds were identified by their mass spectra (Knödler et al., 2008) and quantified using a calibration curve of the corresponding standard compounds.

2.4.4. Determination of fatty acids profile

The fatty acid profile was determined by the AOAC method (AOAC, 1995). The fatty acid methyl esters were firstly prepared and then analyzed by GC-FID (Agilent Technologies, Santa Clara, CA, USA). The separation was carried out with helium (1.8 mL/min) as carrier gas. A fused silica capillary column (Omegawax TM-320, 30 m×0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180 °C for 20 min, heated to 200 °C at 1 °C/min, held at 200 °C for 1 min, heated again to 220 °C at 5 °C/min and finally held at 220 °C for 20 min. A split injector (50:1) at 250 °C was used. The FID was also heated to 250 °C. Fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards. Their quantification was made by relating the peaks area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (AOAC, 1995). Calibration was made for several pairs formed by the internal standard + chromatographic standards in order to find the corresponding response factors.

2.4.5. Determination of antioxidant capacity

FRAP (Ferric Reducing Ability of Plasma) assay

The FRAP assay is used to measure the reductive power of a sample (Benzie & Strain, 1996). It is based on increased absorbance at 593 nm due to the formation of tripyridyl-S-triazine (TPTZ) complexes with ferric (II) in the presence of a reductive agent. Briefly, 970 µL of FRAP reagent was mixed with 30 µL of methanol solutions of bran extracts (5 mg/mL). The FRAP reagent was prepared by mixing 25 mL of 0.3 M sodium acetate buffer solution at pH 3.6, 2.5 mL of TPTZ (10 mM), 2.5 mL of $FeCl_3$ (20 mM), and 3 mL of water. The reaction was carried out at 37 °C during 30 minutes and the absorbance was measured at 593 nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan).

Methanolic solutions of known Fe (II) obtained with different concentrations of $FeSO_4$ were used for calibration.

DPPH assay

Free radical scavenging capacity of wheat bran extracts was evaluated using 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$) (Brand-Williams et al., 1995). Briefly, 980 µL of $DPPH^{\bullet}$ solution (50.7 µM) was mixed with 20 µL of methanol solutions of bran extracts (5 mg/mL). The absorbance at 517 nm was measured (Hitachi U-2000 spectrophotometer, Tokyo, Japan) against a blank of pure methanol after the reaction was carried out at ambient temperature and darkness for 60 min. Methanolic solutions of known Trolox concentrations were used for calibration.

2.5. Statistical analysis

Statistical analysis were performed using a two-way analyses of ANOVA (Statgraphics Centurion XVI.I) and the least significant difference (LSD) test calculated to a significant level of $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1. Yields of conventional solvent extraction and characterization of the extracts

The results corresponding to the conventional solvent extractions are shown in Table 1.2. When acetone was used as solvent (R1 and R3), the shaking method resulted in higher ($p \leq 0.05$) mass of extract but in lower total AR content than the Soxhlet method. According to this result, the antioxidant activity was also higher in the extracts obtained by the Soxhlet method, in spite of the higher temperatures used in this method. This indicates that temperature could be a variable to optimize in the extraction with SC-CO_2.

There was no significant difference ($p \leq 0.05$) between acetone (R1) and ethanol (R2) extracts when comparing the total amount of extract. Zhou and Yu (2004) found that absolute ethanol at room temperature was the solvent least effective among different solvent systems (including 50% acetone solution) for extracting antioxidant agents from wheat bran fractions; while absolute ethanol in Soxhlet was a highly effective extraction method. This confirms the influence of extraction temperature on the antioxidant activity of wheat bran extracts.

When comparing acetone (R3) and petroleum ether (R4) as solvents used in the Soxhlet method, no significant difference ($p \leq 0.05$) were found between the total amount of extract obtained. However, lower levels of total AR content were obtained with petroleum ether. This suggests that non-polar solvents extract fewer AR than more polar solvents

such as acetone. Since AR are amphiphilic compounds, their solubility in non-polar solvents is relatively low. AR could not be quantified by HPLC for the extracts obtained by the shaking method due to experimental problems. In any case, for the rest of the extracts, it must be pointed out that the total content of AR obtained by HPLC analysis is nearly twice than by colorimetric method, concluding that the method used to determine the AR content greatly affects their quantification. Differences may be attributed to the different calibration compounds used for each method; olivetol for the colorimetric method and AR for the HPLC method.

3.2. Influence of process parameters on the extraction yield with SC-CO_2

All the extraction experiments were performed at 40.0 MPa. First, the effect of particle size was determined (Table 1.3). Figure 1.1 shows the extraction curves obtained with bran sieved to three different sizes (R5-R7) and with whole bran (R8). The analysis of the extraction curves shows that the initial extraction rate slightly increases as particle size decreases, but after this initial period, for a fixed extraction time, extraction rates are rather similar. This can be due to the higher amount of compounds that can be extracted outside the particles due to the smaller particle size, which would decrease the importance of diffusion compared to convection (Mezzomo et al., 2009).

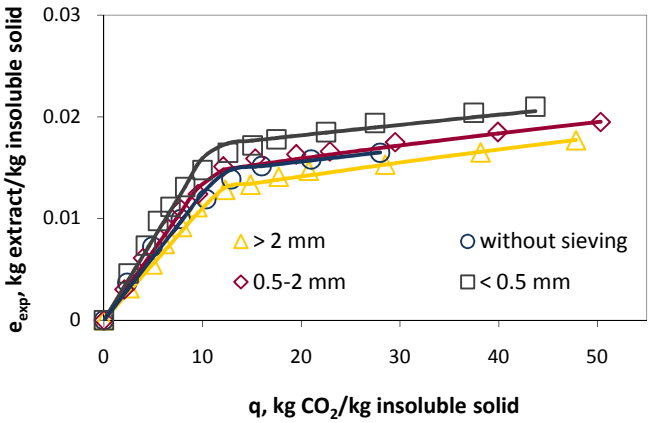

Figure 1.1. Influence of particle size on wheat bran extraction yield at a constant pressure of 40 MPa and at a temperature of 40 °C. The solid lines correspond to the model of Sovová (2005)

Table 1.2. Experimental conditions and results obtained for conventional solvent extraction of wheat bran

Run	Solvent-method	T (°C)	t (h)	mg extract/ g dry bran	µg AR/g dry bran (colorimetric)	µg AR/g dry bran (HPLC)	µmol Trolox/ g dry bran	µmol Fe (II)/g dry bran
R1	Acetone-shaking	20	24	42 ± 12^{a}	1725 ± 82^{a}	-	0.59 ± 0.03^{a}	6.4 ± 0.6^{a}
R2	Ethanol-shaking	20	24	34 ± 1^{ab}	2236 ± 51^{b}	-	0.82 ± 0.07^{b}	5.2 ± 0.5^{a}
R3	Acetone-Soxhlet	56	≈ 3	26 ± 9^{b}	3049 ± 85^{c}	5893 ± 141^{a}	1.59 ± 0.09^{c}	10.4 ± 0.6^{b}
R4	Petroleum ether-Soxhlet	50	≈ 3	24 ± 3^{b}	1287 ± 120^{d}	2217 ± 271^{b}	0.41 ± 0.07^{d}	5.1 ± 0.5^{a}

Values represent mean (n=2) ± standard deviation (SD). Values with different letters in columns are significantly different ($p \leq 0.05$)

Table 1.3. Experimental conditions and results obtained for SFE of wheat bran

Run	p (MPa)	T (°C)	t_c (min)	Raw material	Extraction time (min)	mg extract/ g dry bran	µg AR/g dry bran (colorimetric)	µg AR/g dry bran (HPLC)	µmol Trolox/ g dry bran	µmol Fe (II)/ g dry bran
R5	40.0	40	0	p < 500 µm	150	21.3	448 ± 7	-	-	-
R6	40.0	40	0	p = 0.5-2 mm	215	19 .0	427 ± 3	-	-	-
R7	40.0	40	0	p > 2 mm	185	18.0	440 ± 10	-	-	-
R8	40.0	40	0	without sieving	110	14.3	421 ± 7	-	-	-
R9	40.0	40	60	without sieving	132	18.4	468 ± 10	840 ± 10	0.25 ± 0.01	1.42 ± 0.01
R10	40.0	40	135	without sieving	99	18.1	-	-	-	-
R11	40.0	50	60	without sieving	108	21.5	520 ± 12	912 ± 13	0.29 ± 0.09	2.66 ± 0.03
R12	40.0	60	60	without sieving	210	25.9	618 ± 21	1178 ± 13	0.29 ± 0.06	3.26 ± 0.02
R13	40.0	70	60	without sieving	162	30.6	850 ± 25	1635 ± 36	0.24 ± 0.05	4.06 ± 0.09
R14	40.0	80	60	without sieving	120	34.7	1119 ± 42	2183 ± 86	0.27 ± 0.02	4.91 ± 0.04

When comparing the smallest particle size (< 500 µm, extraction time= 150 min) and the biggest particle size (> 2 mm, extraction time= 185 min) similar amount of AR were obtained in the extracts (Table 1.3). These results can be related to the work of Zarnowski and Suzuki (2004), who stated that in the extraction of AR, grounding of grains is not necessary, because AR are mainly located in a wax cover surrounding the grain. Landberg et al. (2007) also found no difference in AR content or homologue profile in extracts from milled and intact grains when using ethyl acetate as solvent. For the next experiments carried out in this work, wheat bran was used as received, without size screening.

The effect of the exposure time of the wheat bran to SC-CO_2 at the operating pressure without flow of the SC-CO_2 is presented in Figure 1.2 (R8-R10). In general, a static extraction pretreatment leads to a faster extraction of wheat bran. The extraction curves show that a static extraction pretreatment of 60 minutes improve the initial extraction rate, while longer exposure time does not. Similar results were obtained by Ivanovic et al. (2011) in the extraction of essential oil from oregano and thyme. Further extraction experiments to study the effect of extraction temperature were carried out with 60 minutes of exposure time to SC-CO_2.

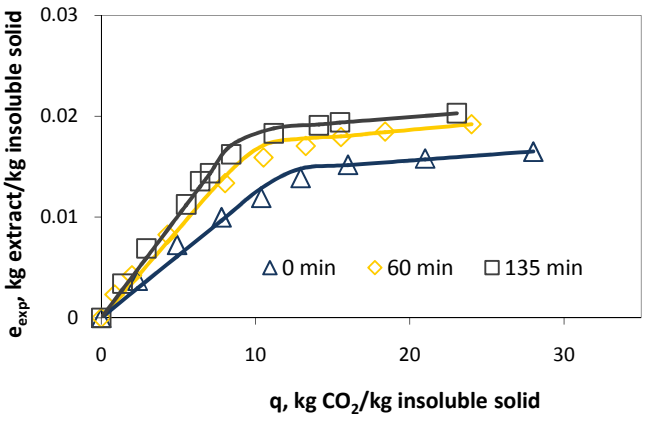

Figure 1.2. Influence of static extraction pretreatment on wheat bran extraction yield at a constant pressure of 40 MPa and at a temperature of 40 °C. The solid lines correspond to the model of Sovová (2005)

The effect of extraction temperature on the extraction yield was evaluated from 40 °C to 80 °C at a constant pressure of 40.0 MPa (runs 11-14 and run 9). The results are shown in

Figure 1.3 where it can be observed that the higher the temperature the higher the extraction rate. This fact indicates that, the increase of temperature increases the vapor pressure of the components to be extracted compensating the depletion in SC-CO_2 density. At a fixed extraction time, the extraction yield increases when temperature increases. Analysis of the extracts showed that the total AR content increased when the extraction temperature was increased (Table 1.3).

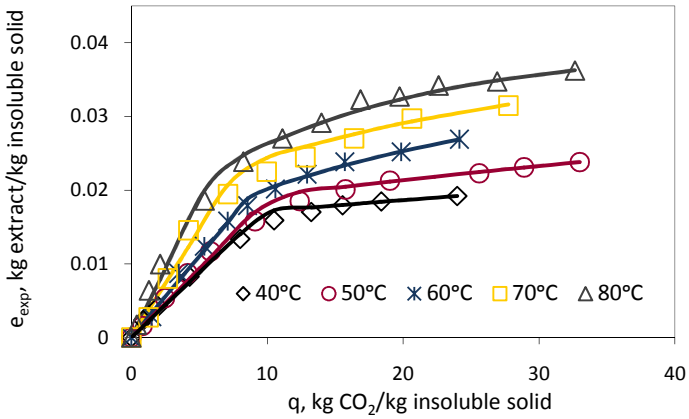

Figure 1.3. Influence of extraction temperature on wheat bran extraction yield at a constant pressure of 40 MPa. The solid lines correspond to the model of Sovová (2005)

3.3. Modeling of the supercritical fluid extraction

In this work, the approximate model proposed by Sovová (2005) was used to describe the experimental extraction curves. This type of model assumes that the solute is regarded as a single pseudo compound. This simplification can lead to some errors since several components are generally involved in the extraction of complex mixtures. Additionally, according to Sovová (2012), the differences in the solubility of extract components can lead to time fractionation as it has been observed in the SFE of essential oils, that is, the more soluble components were preferentially extracted at the beginning and the least soluble components were found in higher concentrations in the extract samples from the final extraction period. In this work, the mathematical modeling of the extraction curves was done for the wheat bran extract, instead of the AR, since these compounds represent only a low portion of the extract.

In the model of Sovová, the extraction yield is expressed as:

$$e = \frac{E}{N_m} \quad \text{(Eq.1.1)}$$

where E is the amount of extract (kg) and N_m the charge of insoluble solid (kg) in the extractor. The dimensionless amount of solvent consumed is obtained by:

$$q = \frac{Qt}{N_m} \quad \text{(Eq.1.2)}$$

where Q is the solvent flow rate (kg/h) and t the extraction time (h). Based on this model, the extraction curves consist of two parts. During the first one, the easily accessible solute from broken cells is transferred directly to the fluid phase, while in the second one the solute from intact cells diffuses first to broken cells and then to the fluid phase. This leads to extraction curves with two parts each corresponding to these two mass transfer processes. When solute-matrix interactions take place, the solute never saturates the fluid phase and a smooth transition appears between the first part of the extraction curve and its end (Martín et al., 2011). Equations (1.3) and (1.4) proposed by Sovová (2005) were used to evaluate the first and second part of the extraction curve respectively:

$$e = q\frac{KX_u}{1+K(\gamma/r)} = qy_o, \text{ for } 0 \leq q \leq q_c \quad \text{(Eq.1.3)}$$

$$e = x_u[1 - C_1 \exp(C_2 q)], \text{ for } q > q_c \quad \text{(Eq.1.4)}$$

where y_o is the slope of the linear part of the curve when the experimental extraction yield, e_{exp}, is plotted vs q, which represent the initial fluid-phase concentration kg solute·kg solvent^{-1}, q_c is the relative amount of the passed solvent when all the solute in broken cells has been extracted, r is the volumetric fraction of broken cells in the particles, so called grinding efficiency, γ is the solvent-to-matrix ratio in the bed (Sovová, 2005):

$$\gamma = \frac{\rho_f \varepsilon}{\rho_s (1-\varepsilon)} \quad \text{(Eq. 1.5)}$$

Where ρ_f is the fluid density at the operating temperature and pressure, ρ_s is the bulk density and ε the bed porosity. The model has two adjustable parameters C_1 and C_2. The partition coefficient, K, and r are obtained simultaneously in the fitting procedure.

$$r = 1 - C_1 \exp(-C_2 q_c) \quad \text{(Eq.1.6)}$$

The constants C_1 and C_2 of the model were obtained by minimizing the root squared mean deviation between experimental and calculated yield (Langa et al., 2009):

$$O.F. = \frac{\sum_{i=1}^{n}[e_{exp} - e_{calc})/e_{exp}]_i^2}{n} \quad \text{(Eq.1.7)}$$

by using the Simplex-Nelder-Mead method. x_u is the solute concentration in the untreated solid, kg solute/kg insoluble solid. In this work, x_u was evaluated numerically by entering it as an adjustable parameter. As suggested by Martín et al. (2011) it would not have been appropriate to obtain it from other extraction methods, in our case solvent extraction methods, due to the different composition of the extracts. In any case, it must be noticed that the initial value used in the fitting procedure greatly affects the value obtained for x_u. In this work, the initial value considered was the value obtained by extrapolation of the experimental mass of extract vs time under the most favorable conditions used in this work (Martínez et al., 2003).

The calculated extraction curves are plotted in Figures 1.1 to 1.3. From these Figures, a good agreement can be observed between experimental data and model correlation. The mean relative deviations (MRD) between experimental and calculated yields were calculated for each extraction curve:

$$MRD = \frac{1}{n}\sum_{i=1}^{n} abs\left(\frac{e_{exp} - e_{calc}}{e_{exp}}\right)_i * 100 \quad \text{(Eq.1.8)}$$

Fitting parameters and the values of the mean relative deviation along with some mass transfer parameters are collected in Table 1.4. This Table also shows the $k_s a_s$ values calculated according to the approximate model of Sovová (2005):

$$K_s\, a_s = \frac{(1-r)(1-\varepsilon)QC_2}{N_m[1-((1-r)C_2/K)]} \quad \text{(Eq.1.9)}$$

The value of x_u obtained in the fitting procedure remains more or less constant in all the SFE experiments and above the value reached in any of the SFE experiments. The grinding efficiency, r, increases as the particle size decreases indicating that there are probably more broken cells. The same tendency was observed by Langa et al. (2009) in the SFE of Spanish sage essential oil and by Grosso et al. (2010) in the SFE of volatile oils from different aromatic plants. In contrast, $k_s a_s$ remains more or less constant in the particle size range covered in this work. At constant pressure, $k_s a_s$ increases with temperature although some authors (Martín et al., 2011) found it to increase with CO_2 density.

Table 1.4. Values of the parameters obtained with the approximate model of Sovová (2005) and MRD for each experiment

Run	y_o	K	r	x_u	$k_s a_s$	C_1	C_2	q_c	MRD
R5	0.00160	0.062	0.44	0.0386	$4.5 \cdot 10^{-6}$	0.5869	0.0052	10.0	4.5
R6	0.00136	0.063	0.42	0.0344	$4.0 \cdot 10^{-6}$	0.6215	0.0072	9.6	3.6
R7	0.00111	0.048	0.34	0.0371	$6.6 \cdot 10^{-6}$	0.6993	0.0061	9.5	3.0
R8	0.00124	0.051	0.39	0.0370	$4.5 \cdot 10^{-6}$	0.6429	0.0053	10.4	6.8
R9	0.00175	0.069	0.45	0.0375	$4.8 \cdot 10^{-6}$	0.5845	0.0075	8.0	8.7
R10	0.00203	0.096	0.49	0.0375	$3.2 \cdot 10^{-6}$	0.5393	0.0070	8.4	4.6
R11	0.00186	0.072	0.48	0.0396	$1.1 \cdot 10^{-5}$	0.5754	0.0111	9.1	11.8
R12	0.00220	0.093	0.54	0.0354	$2.0 \cdot 10^{-5}$	0.6496	0.0412	8.6	4.5
R13	0.00291	0.113	0.59	0.0385	$2.9 \cdot 10^{-5}$	0.5496	0.0404	7.2	8.6
R14	0.00367	0.162	0.55	0.0395	$5.7 \cdot 10^{-5}$	0.6286	0.0626	5.4	6.4

3.4. Characterization of wheat bran extracts

3.4.1. Fatty acid profile

Extracts of wheat bran obtained with SC-CO_2 contain other compounds apart from the target AR. The main non-polar lipids compounds in the extracts of wheat bran with SC-CO_2 were fatty acids (607 ± 32 mg/g extract).

The fatty acid profile (Table 1.5) was mainly composed by polyunsaturated fatty acids (around 60%). The majority fatty acid is linoleic acid (C18:2 n-6) followed by oleic acid (C18:1 n-9). Within saturated fatty acids, palmitic acid (C16:0) was the most common acid. Fewer amounts of α- linolenic and stearic acids were also presented. Table 1.5 also reports fatty acid composition of other SC-CO_2 extracts of wheat bran found in the literature (Athukorala et al., 2010; Durante et al., 2012; Kwon et al., 2010). In all cases, the majority fatty acid is linoleic acid (C18:2 n-6) followed by oleic acid (C18:1 n-9).

Table 1.5. Fatty acid profile (g/100 g fatty acids) of wheat bran oil obtained with SC-CO_2

Fatty acid	This work (R9-R14)	Durante et al. (2012)	Kwon et al. (2010)	Athukorala et al. (2010)
Palmitic acid, C16:0	16.9 ± 0.2	19.2 ± 0.3	15.5 - 22.0	21 ± 1
Stearic acid, C18:0	1.9 ± 0.1	1.0 ± 0.1	-	5.0 ± 0.8
Oleic acid, C18:1 n-9	17.4 ± 0.4	27.8 ± 0.7	11.8 – 15.9	22 ± 2
Linoleic acid, C18:2 n-6	56 ± 1	51 ± 1	45.4 – 57.3	46 ± 6
α-linolenic acid, C18:3 n-3	5.8 ± 0.1	1.4 ± 0.1	5.7 -8.0	6.0 ± 0.6
Others	2.0 ± 0.1	0.15 ± 0.01	-	-

3.4.2. Comparison of extracts obtained with SC-CO_2 and organic solvents: AR content and AR profile

Extracts obtained using acetone and petroleum ether as solvents by Soxhlet method (R3 and R4) and some of the extracts obtained by SC-CO_2 extraction were analyzed by HPLC to determine the AR profile (Figure 1.4). No significant differences can be observed in the AR homologue composition of the extracts obtained by conventional solvent extraction and the extracts obtained by SC-CO_2 extraction. C21:0 homologue has been found to be the most abundant, nearly 50%, followed by C19:0 (25%). Although several factors, such as genetic factors, climate, season, grain filling period and soil conditions can affect the AR content (Athukorala et al., 2010), similar AR profile was also found by Landberg et al. (2007) in the extraction of wheat bran (milled) by using ethyl acetate and SC-CO_2.

Even though similar AR profile was obtained by conventional organic solvent extraction and by SC-CO_2 extraction, it must be pointed out that highest AR/extract ratio is obtained when using organic solvents, mainly polar, as acetone. From Table 1.2, it can be easily obtained that the AR content of the extract obtained with acetone (R3) represents as much as 22% (by HPLC). This percentage decreases down to 9% (by HPLC) when using ether petroleum (R4) as organic solvent. This fact, as it was previously explained, is due to the amphiphilic nature of AR compounds. However the percentage of AR obtained when using SC-CO_2 is only 6.5% at the highest temperature studied in this work. Landberg et al. (2007) reported similar values of AR content in dry extract of wheat bran

(milled) with ethyl acetate (5.7 ± 0.2%) and with SC-CO_2 (6.2 ± 0.4%). In spite of the higher AR yield obtained by Soxhlet acetone method compared to SC-CO_2, solvent extraction presents some disadvantages including long extraction times, toxic waste generation and a more laborious final purification process.

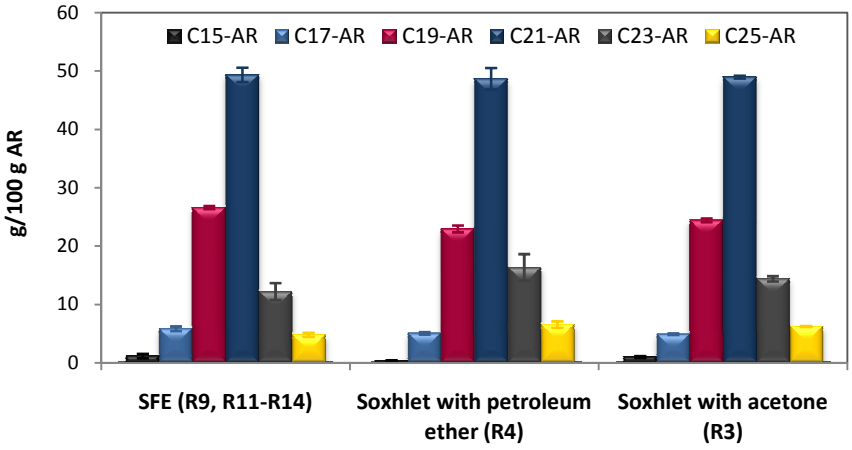

Figure 1.4. AR profile obtained by HPLC for different extraction methods and solvents

3.4.3. Antioxidant activity of wheat bran extracts

The relationship between the antioxidant activity measured by the FRAP method and the AR content of the extracts obtained under the different extraction conditions (see Tables 1.2 and 1.3) suggests that the antioxidant mechanism of AR is based on single electron transfer (SET) reactions. Also, an increase of the antioxidant activity evaluated by the DPPH method was observed when the AR content increased in the extracts obtained with organic solvents (Table 1.2) what is in agreement with Korycinska et al. (2009) who reported a clear relationship between antioxidant activity of breakfast cereal extracts and their total amount of AR.

4. CONCLUSIONS

SC-CO_2 extraction has been studied to obtain extracts from wheat bran fraction. The influence on extraction yield and extraction quality of some SFE parameters, such as particle size, static extraction pretreatment (0-135 min), and extraction temperature (40-80 °C) at a constant extraction pressure of 40.0 MPa was studied. Temperature is one

of the most important parameters on the extraction yield, obtaining high amount of extract as well as more AR content and antioxidant capacity when the extraction temperature was 80 °C. The extraction curves were well represented by the approximate model of Sovová (2005).

The SC-CO_2 wheat bran extract has an important content in fatty acids, mainly polyunsaturated, being linoleic acid the majority followed by oleic acid. In general, SC-CO_2 extraction applied to wheat bran results in a lipophilic extract with appreciable AR content and antioxidant capacity. Due to the amphiphilic nature of AR compounds the ratio AR/extract was higher when extraction was performed with polar organic solvents such as acetone. It can be concluded that a valuable extract rich in AR has been obtained by SC-CO_2 extraction from a by-product such as wheat bran fraction.

Nomenclature

a_s = specific area between the regions of intact and broken cells (m^{-1})

C_1, C_2 = fitting parameters

e = extraction yield (kg extract·kg insoluble $solid^{-1}$)

E = extract (kg)

k_s = solid-phase mass transfer coefficient (s^{-1})

K = partition coefficient

MRD = mean relative deviation

n = number of experimental data

N_m= charge of insoluble solid (kg)

O.F. = objective function

Q = solvent flow rate ($kg \cdot h^{-1}$)

q = relative amount of the passed solvent (kg solvent·kg insoluble $solid^{-1}$)

q_c = relative amount of the passed solvent when all the solute in broken cells has been extracted (kg solvent·kg insoluble $solid^{-1}$)

r = grinding efficiency (fraction of broken cells)

t = extraction time (h)

x_u = concentration in the untreated solid (kg solute·kg solid $insoluble^{-1}$)

y_s = solubility (kg solute·kg $solvent^{-1}$)

ρ = density ($kg \cdot m^{-3}$)

ε = porosity

γ = solvent to matrix ratio in the bed (kg solvent·kg insoluble $solid^{-1}$)

Subscripts:

exp = experimental

calc = calculated

f = fluid

s = solid

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Chapter 2

Supercritical fluid extraction of wheat bran oil: optimization of extraction yield and oil quality

Based on the article:

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ABSTRACT

The supercritical fluid extraction of wheat bran oil with pure supercritical carbon dioxide in a pilot plant at different extraction pressure (25, 40 and 55 MPa) and temperature (40, 70 and 95 °C) has been studied. Since wheat bran is characterized by having an important content of bioactive compounds such as alkylresorcinols (AR) and other phenolic compounds, the content of such compounds in the extracted oil was evaluated in order to establish oil quality. The influence of extraction pressure and temperature and wheat bran moisture on the extraction yield and oil quality was studied. Oil quality was evaluated through parameters such as AR content and profile, total polyphenols index, antioxidant activity and fatty acids profile. SFE performed over fresh wheat bran at 55 MPa and 95 °C was found to provide wheat bran oil with the highest AR and phenolic content and antioxidant capacity of all the runs performed in this work. Some SFE experiments focused on oil fractionation showed that this technique can provide oil fractions enriched with AR and phenolic compounds.

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1. INTRODUCTION

Wheat bran oil containing alkylresorcinols (AR) has been successfully extracted with pure supercritical CO_2 (SC-CO_2) operating in a laboratory plant (Chapter 1) (Rebolleda et al., 2013). In the present chapter, the extraction of wheat bran oil in a supercritical fluid extraction (SFE) pilot plant has been studied. The pilot plant used can work at higher pressure (up to 65 MPa) and it is provided with a higher extractor volume (2 L) than the previously used laboratory plant (Appendix A).

SFE of wheat bran has been less reported than rye bran extraction but some studies on wheat bran oil quality have shown the oil obtained by SFE to have a slightly better quality than hexane extracted oil in terms of acidity, peroxide value and antioxidant activity (Jung et al., 2012). Most of the previous works on SFE applied to cereal bran have dealt with the use of SC-CO_2 as solvent together with some cosolvents that help to modify the polarity of the solvent system (Francisco et al., 2005a, 2005b; Landberg et al., 2007). In some cases, AR have been concentrated using two stage SC-CO_2-extraction, a first SFE stage with pure SC-CO_2 (Athukorala et al., 2010) or modified with very low amount of ethanol (0.06%) (Dey & Mikhailopulo, 2009) for removing the non-polar lipids, and a second SFE stage using ethanol as cosolvent of the CO_2 for obtaining a polar fraction where AR were concentrated (Athukorala et al., 2010; Dey & Mikhailopulo, 2009).

The aim of this chapter was to study the quality of the wheat bran oil obtained by using pure SC-CO_2 under different conditions as extraction solvent. Parameters such as AR content and profile, total polyphenols index (TPI), antioxidant activity, and fatty acids profile were evaluated. Additionally, the influence of some process parameters, such as pressure and wheat bran moisture, not evaluated in Chapter 1 were addressed in this work. Also the range of extraction temperatures studied was increased in this chapter since previously (Chapter 1) this parameter was shown to have a significant influence on the extraction yield and oil quality. Finally, a two-stage SFE with pure CO_2 under different extraction conditions was carried out.

2. MATERIALS AND METHODS

2.1. Raw material

The raw material used in this work was wheat bran (T*riticum aestivum L.*) kindly provided by HASENOSA (Spain). Fresh and dried bran were used. Fresh bran was dried in an oven at 70 °C during 48 h for those experiments that required dried bran.

2.2. Solvents and reagents

Liquid CO_2 (≥ 99.9%) was provided by Carburos metálicos (Barcelona, Spain), methanol (≥ 99.9%) by LabScan (Gliwice, Poland), ethanol (≥ 99.9%) by Merck (Darmstadt, Alemania), Fast Blue RR salt, olivetol, AR standards, ABTS (2,29-azinobis (3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and fatty acid methyl esters by Sigma-Aldrich Co. (St. Louis, MO, USA), potassium carbonate (K_2CO_3) and potassium persulphate ($K_2O_8S_2$) by Panreac (Barcelona, Spain) and methyl tricosanoate by Larodan (Malmö, Sweeden).

2.3. Supercritical fluid extraction equipment and procedure

The extraction experiments were carried out in a semi-pilot SFE-plant (Appendix A). In a SFE experiment, 300 g of wheat bran were placed in the extractor (2 L capacity) that was later pressurized with CO_2 up to the extraction pressure. Then, the solvent was circulated at the desired extraction temperature, *T*, with a solvent flow of 9 ± 1 kg CO_2/h and during an extraction time of 120 min. This extraction time was chosen based in the results obtained in Chapter 1 to circulate enough amount of SC-CO_2 for completing extraction of the soluble compounds. The solvent was continuously recycled to the extractor after removing the solute in the separator that was kept at 4.9 ± 0.6 MPa and 24 ± 2 °C.

Co-extracted water was removed from the extracts by vacuum evaporation at 45 °C during 90 min using a Heidolph VV2000 rotary evaporator (Schwabach, Germany). Oils were stored at -20 °C until analysis.

Table 2.1 shows the experimental conditions used to evaluate the influence of wheat bran moisture (R1 and R6) and extraction pressure and temperature (R2 to R10) on the oil yield and quality. A two-stage extraction (R11) was carried out in order to obtain a rich AR oil fraction. The extraction time for each stage was 60 min. The rest of the extraction conditions for each stage are shown in Table 2.1. All the runs were performed in triplicate and the results are given as average values ± standard absolute deviation.

Table 2.1. Experimental runs of SFE of wheat bran with pure SC-CO_2

Run	Extraction conditions		
	P (MPa)	T (°C)	Wheat bran moisture (%)
R1	41 ± 2	70 ± 1	1.6 ± 0.3
R2	25 ± 1	40 ± 1	11.0 ± 0.1
R3	25 ± 1	70 ± 1	11.0 ± 0.1
R4	25 ± 1	95 ± 1	11.0 ± 0.1
R5	40 ± 2	40 ± 1	11.0 ± 0.1
R6	40 ± 2	70 ± 1	11.0 ± 0.1
R7	40 ± 2	95 ± 1	11.0 ± 0.1
R8	55 ± 2	40 ± 1	11.0 ± 0.1
R9	55 ± 2	70 ± 1	11.0 ± 0.1
R10	55 ± 2	95 ± 1	11.0 ± 0.1
R11 (step 1)	25 ± 1	40 ± 1	11.7 ± 0.1
R11 (step 2)	50 ± 2	83 ± 2	-

Data are given as mean values ± SD; number of replicas n = 3

2.4. Analytical methods

A full description of the analytical methods used in this chapter can be found in the Appendix B.

2.4.1 Determination of total AR

The total AR content in the extracted material was determined by a colorimetric method based on the use of Fast Blue RR salt (Sampietro et al., 2009). A stock solution of 0.05% Fast Blue RR reagent was used to prepare a working solution by mixing 1 part of stock reagent with 4 parts of methanol. Aliquots (20 µL) of ethanol solution of wheat bran oil (5 mg/mL) were placed in assay tubes and made up to 200 µL with ethanol. Then, 2 mL of the working solution and 10 µL of a 10% K_2CO_3 solution were also added to each tube. Absorbance of the reaction mixture was measured at 480 nm after 20 min in a Hitachi U-

2000 spectrophotometer (Tokyo, Japan). Olivetol (5-pentylresorcinol) was used as standard.

2.4.2 Determination of AR profile

Alkylresorcinols were determined according to a modification of Knödler et al. (2008) method using an Agilent HPLC (series 1100) equipped with a diode array detector and a mass spectrometry detector with an APcI source. The column used was Kromasil C18-5 250 x 4.6 mm and operated at 25 °C. The mobile phase was methanol (A) and water (B) and the following gradient was used: 2% B to 0% B in 10 min. The total run time was 100 min. 100 µL of methanolic solutions of wheat bran oil (10 mg/mL) were injected**.** All AR were monitored at 280 nm at a flow rate of 0.6 mL/min.

Positive-ion mass spectra of the column eluate compounds were recorded in the range m/z 100–500. Nitrogen was used both as the drying gas at a flow rate of 10 L/min and as the nebulizing gas at a pressure of 380 Pa. The nebulizer temperature was set at 350 °C and a potential of 4000 V was used on the capillary.

Individual compounds were identified by their mass spectra (Knödler et al., 2008) and quantified using a calibration curve of the corresponding standard compounds.

2.4.3 Determination of Total Polyphenol Index (TPI)

The TPI is based on the maximum absorbance that many phenolic compounds present at 280 nm. Ethanol solutions of wheat bran oil (5 mg/mL) were diluted with ethanol (1:10) and absorbance at 280 nm was measured in a Hitachi U-2000 spectrophotometer (Tokyo, Japan).

2.4.4 Determination of antioxidant capacity: ABTS assay

This assay is based on decolorization that occurs when the radical cation $ABTS^{\bullet+}$ is reduced to ABTS´ (Re et al., 1999). The radical was produced by reaction of 7 mM solution of ABTS with 2.45 mM $K_2O_8S_2$ (1:1). This mixture was kept in darkness at room temperature for 16 hours before use in order to obtain a stable radical $ABTS^{\bullet+}$ solution (Rivero-Pérez et al., 2007).

The assay was made with 980 µL of diluted $ABTS^{\bullet+}$ and 20 µL of ethanol solution of bran oil (5 mg/mL). The absorbance at 734 nm was measured after 20 min of reaction in a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Ethanolic solutions of known Trolox concentrations were used for calibration.

2.4.5 Determination of fatty acids profile

The fatty acids profile was determined by the AOAC method (AOAC, 1995). The fatty acid methyl esters were firstly prepared and then analyzed by GC-FID (Agilent Technologies, Santa Clara, CA, USA). The separation was carried out with helium (1.8 mL/min) as carrier gas. A fused silica capillary column (Omegawax TM-320, 30 m×0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180 °C for 20 min, heated to 200 °C at 1 °C/min, held at 200 °C for 1 min, heated again to 220 °C at 5 °C/min and finally held at 220 °C for 20 min. A split injector (50:1) at 250 °C was used. The FID was also heated to 250 °C. Fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards. Their quantification was made by relating the peaks area to the area of an internal standard (methyl tricosanoate) and using the corresponding chromatographic standards in order to find the response factors of each compound, as indicated by the AOAC method (AOAC, 1995).

3. RESULTS AND DISCUSSION

3.1. Effect of wheat bran moisture on the extraction yield and oil quality

Table 2.2 shows the extraction yield and quality of the oil obtained by SFE from dry and fresh wheat bran under the same extraction conditions (40 MPa and 70 °C). A reduction of 40% can be observed for the total extraction yield when dry bran was used. This reduction might be explained by the role that water plays during the extraction process of plant materials. The lipid pillars of an elementary membrane change with water content. If there is not enough water in the system, the pillars may close the membrane, making it impermeable, and therefore hindering solute extraction (Brunner, 1994). The lower yield obtained when extracting dry bran, also resulted in a lower extraction of AR and polyphenols, which corresponded with a minor antioxidant capacity of the extract. Since TPI is similar in both extracts, the lower antioxidant capacity of the extract obtained from dry bran can be attributed to the reduction in the AR content.

Table 2.2. Characterization of the wheat bran oil obtained by SFE at 40 MPa and 70 °C from dried and fresh wheat bran

		R1	R6
Wheat bran moisture (%)		1.6 ± 0.3	11.0 ± 0.1
Extraction yield (g oil/100 g dry bran)		1.8 ± 0.1	2.9 ± 0.2
AR content (mg/g oil)	Colorimetric method	31 ± 2	53 ± 3
	HPLC method	62 ± 3	138 ± 5
TPI (abs units/g oil)		857 ± 35	928 ± 28
ABTS (mmol Trolox/g oil)		0.32 ± 0.01	0.39 ± 0.05
Fatty acids (mg/g oil)		815 ± 15	786 ± 15

Data are given as mean values ± SD; number of replicas n = 3

The AR profile of the extracts obtained from dry and fresh wheat bran (R1 and R6) was also determined by HPLC-DAD. The AR profile and one of the chromatograms are given in Table 2.3. Similar profile was determined for both oils although it slightly differs from those reported in the literature where C15:0 is not always identified (Andersson et al., 2008; Ross et al., 2003). These differences might be explained by the different calibration methods used. The different compound used for calibration can also explain the differences obtained in this work between the colorimetric method with Fast Blue RR, calibrated with olivetol, and the HPLC method calibrated using several AR.

AR composition and the ratio of the AR homologues C17/C21 are especially useful in distinguishing cereals species. Our results corroborate those obtained by Chen et al. (2004) and Andersson et al. (2008) who reported that the C17/C21 ratio in common wheat is 0.1. In contrast, Mattila et al. (2005) and Kulawinek et al. (2008) obtained a higher C17/C21 ratio for wheat samples, which they attributed to unresolved AR in the HPLC.

Taking into account the higher extraction yield and AR content achieved with fresh bran, it was used for the next experiments performed in this work.

Table 2.3. Alkylresorcinols content in wheat bran oil as determined by HPLC-DAD

Alkyl chain	Oil AR content (mg/g oil)		Oil AR profile (g/100 g AR)		
	R1	R6	R1	R6	*
C15	0.99 ± 0.05	1.40 ± 0.04	1.6 ± 0.1	1.0 ± 0.1	0-1
C17	3.7 ± 0.2	7.6 ± 0.3	6.0 ± 0.4	5.5 ± 0.3	4-9
C19	13.70 ± 0.07	33.6 ± 0.6	22 ± 1	24.5 ± 0.9	31-35
C21	33 ± 3	73 ± 3	54 ± 2	52.4 ± 0.8	45-50
C23	7.0 ± 0.2	16.4 ± 0.7	11.2 ± 0.2	11.8 ± 0.4	8-10.5
C25	3.33 ± 0.07	6.6 ± 0.3	5.3 ± 0.1	4.8 ± 0.1	2-4

Data are given as mean values ± SD; number of replicates n=3.

*Chen et al. (2004); Kulawinek et al. (2008); Ross et al. (2003)

3.2. Effect of process variables on oil extraction yield

The effect of temperature on the extraction yield was evaluated at 40, 70 and 95 °C at three different pressures (25, 40 and 55 MPa) and a constant flow of 9 ± 1 kg CO_2/h. The results are shown in Figure 2.1. At the lowest pressure used in this work, 25 MPa, it appears not to be a significant effect of temperature on the total amount of oil obtained, while at the two other pressures evaluated, 40 and 55 MPa, the extraction yield increases with temperature, which may indicate that, at these pressures, the increase of oil vapor-pressure with temperature is more important than the decrease of SC-CO_2 density. These results suggest a crossover behavior of the isotherms around 25 MPa. This behavior has not been described in the literature for wheat bran oil but it has been generally observed for different oils (Özkal et al., 2005). Due to the possibility of a crossover region around 25 MPa, influence of a pressure increase seems to be stronger at the highest temperature evaluated in this work.

The extraction yield obtained at 40 MPa and 70 °C (R6) was 2.9 ± 0.2 g oil/100 g dry bran. This result is similar to that reported by Athukorala et al. (2010) who obtained approximately 3.3 ± 0.5 g/100 g wheat bran, when operating at 35 MPa and 70 °C with pure SC-CO_2. Landberg et al. (2007) obtained 4.9 ± 0.5 g oil/100 g dry matter when they extracted wheat bran with SC-CO_2 and ethanol as cosolvent, at 35 MPa and 70 °C. The

higher yield obtained under these extraction conditions is due to the use of a cosolvent that modifies the polarity of the solvent mixture. Francisco et al. (2005a) also obtained higher yields in the extraction of rye bran when ethanol or methanol were used as cosolvents than when using pure CO_2.

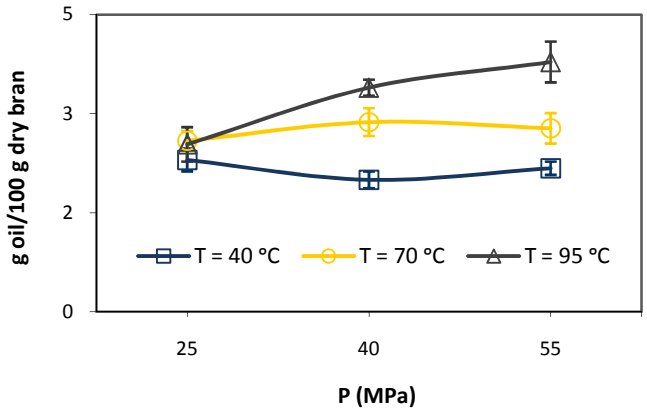

Figure 2.1. Influence of extraction pressure and temperature on total oil yield (R2 – R10). Lines are to guide the eye

Oils presented different visual appearance depending on extraction conditions (Figure 2.2). When high extraction pressures and temperatures were used, some emulsification of the oil and the coextracted water seemed to take place in the separation stage. On the one hand, the higher the pressure the higher the pressure drop taking place in the expansion performed for separation of the extract and the CO_2 leading a vigorous mixing between oil and the coextracted water in the separator. On the other hand, water solubility in CO_2 is highly affected by the extraction temperature and pressure and therefore more water is coextracted when higher temperatures and pressures were used.

As the *vacuum* evaporation of the coextracted water was not complete in emulsified oils, the final water content in these samples was determined by drying them in an oven at 105 °C until constant weight and considered in the analytical results obtained in this work.

It should be noted that in the experimentation carried out in the laboratory plant (Chapter 1), the coextracted water was dragged with the CO_2 as it was not recirculated and no emulsificated extracts were obtained.

Figure 2.2. Example of the oils obtained at 25 MPa and 40 °C (R2, left) and 55 MPa and 70 °C (R7, right)

3.3. Effect of process variables on oil quality

3.3.1 Effect of process variables on oil AR content

The total AR content of the oils obtained by SFE under the different extraction pressures and temperatures was evaluated using the colorimetric method described in Section 2.4. The results are shown in Figure 2.3 where it can be observed that the amount of AR in oil slightly increased with extraction temperature when extraction pressure was 40 and 55 MPa. However, at 25 MPa, there was not significantly effect of the extraction temperature on the AR oil content. This suggests a crossover behavior around 25 MPa for AR extraction, similar to that found for oil extraction in section 3.2. Higher temperatures seem to provide not only higher extraction yields but also oil with higher AR content. Francisco et al. (2005a) studied the extraction of rye bran with SC-CO_2 + cosolvents at pressures of 8, 15, 30 and 35 MPa and temperatures of 40 and 55 °C. They found that the extractability of AR slightly decreased with temperature when using extraction pressures of 8 and 15 MPa while it increased when working under pressures of 30 and 35 MPa. They attributed this behavior to the presence of a crossover region between 15 and 30 MPa. In a later work, Francisco et al. (2005b) concluded that the influence of the extraction temperature on the extraction of AR from rye bran when using SC-CO_2 and ethanol as cosolvent is not significant; nevertheless, according to the results obtained in this work, we might suggest that the extraction temperature in the range of 40 to 95 °C has a significant influence on wheat bran SFE with pure CO_2.

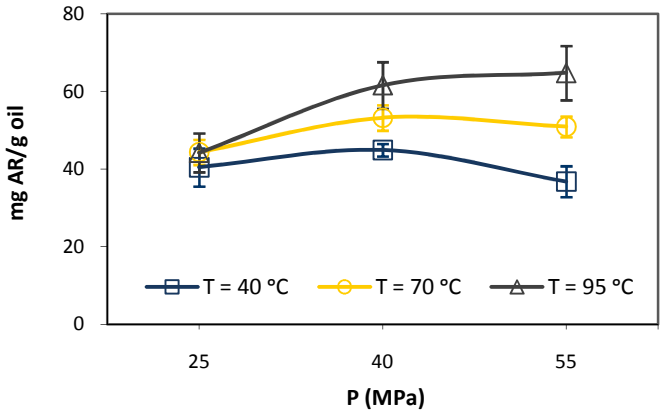

Figure 2.3. Influence of extraction pressure and temperature on AR content of wheat bran oils (R2 - R10). Lines are to guide the eye.

The total AR content of the oils obtained under the different extraction conditions varied from 37 to 65 mg AR/g oil as evaluated by the colorimetric method (see Fig. 2.3). These results are similar to those reported by Landberg et al. (2007) who obtained an extract with 62 mg AR/g extract when operating at 35 MPa, 70 °C and using ethanol as cosolvent. A comparison of both results may indicate that the absence of cosolvent could be offset with higher extraction temperatures and pressures. Francisco et al. (2005a, 2005b) and Athukorala et al. (2010) reported that pure SC-CO_2 could not isolate the AR homologues from rye and wheat bran at 35 MPa and 70 °C. Nevertheless, the results found in this work showed the viability of AR extraction using pure CO_2.

3.3.2 Effect of process variables on oil TPI

Wheat bran presents high levels of total phenolic acids (around 4.5 g/kg dry bran) mainly linked to different cell-wall materials being the major phenolic compound ferulic acid (Mattila et al., 2005). The total polyphenolic content of the extracts was evaluated through the Total Polyphenol Index (TPI) as described in Section 2.4. TPI gives an indication of the amount of polyphenols in the SFE oil. Figure 2.4 shows the TPI for all the oils evaluated.

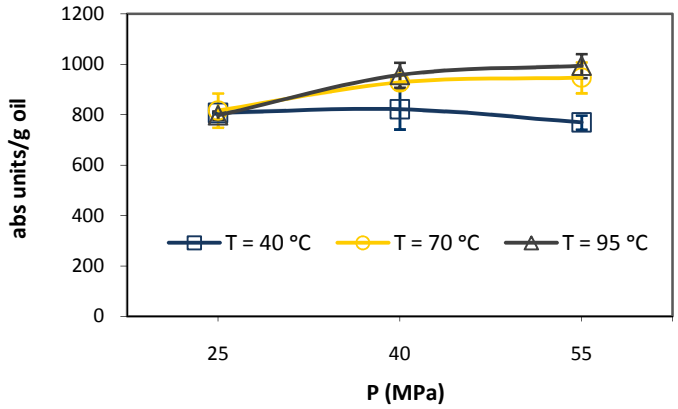

Figure 2.4. Influence of extraction pressure and temperature on total polyphenol index of wheat bran oil (R2-R10). Lines are to guide the eye

TPI seems not to be highly affected by extraction pressure or temperature although some slight increase can be observed, mainly with temperature. The influence of a pressure increase seems to be stronger at the highest temperatures evaluated in this work, what may be indicating the presence of a crossover region in phenols solubility. In fact, the solubility of some phenols in pure SC-CO_2 has been previously studied (Murga et al., 2002, 2003; Murga et al., 2004). Solubility values for ferulic acid, for instance, present crossover behavior around 20 MPa. This is a fairly common behavior for other phenolic compounds and is probably determining the influence of the extraction parameters on TPI.

3.3.3 Effect of process variables on oil antioxidant capacity

The antioxidant capacity of the oil obtained under different extraction conditions was measured by the ABTS assay which is applicable to both hydrophilic and lipophilic antioxidants (Re et al., 1999). Figure 2.5 reports the antioxidant capacity expressed as Trolox equivalent for the different oils obtained in this work. The influence of extraction temperature and pressure was similar to that reported above for the AR content of the extract. Relationship between the antioxidant capacity of the extract from breakfast cereals and their total amounts of AR was previously reported by Korycinska et al. (2009). Some similarity can be also observed between the effect of pressure and temperature on TPI (Fig. 2.4) and on antioxidant capacity evaluated by the ABTS method (Fig. 2.5) indicating some correlation between both parameters. Correlation between total phenolic

content and the ABTS scavenging capacity was previously found by Zhou et al. (2004) in wheat extracts.

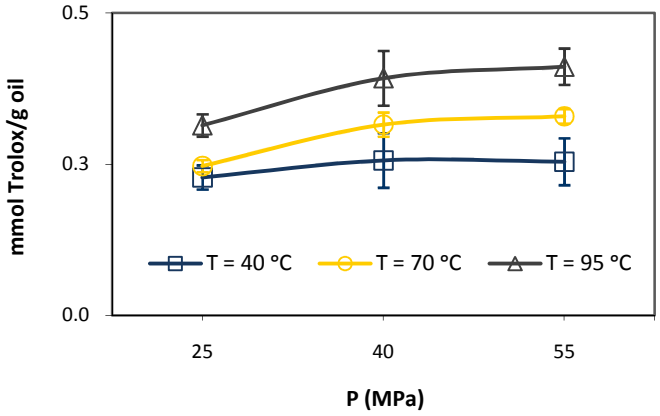

Figure 2.5. Influence of extraction pressure and temperature on $ABTS^{\bullet+}$ scavenging capacity of wheat bran oils expressed as mmol Trolox equivalents. Lines are to guide the eye

Antioxidant capacity varies from 0.23 to 0.41 mmol Trolox/g oil (5.3-15.6 µmol Trolox/g dry bran) depending on the extraction conditions (see Fig. 2.4). The highest antioxidant capacity was obtained when the highest temperature and pressure were used. These values were of the same order as those reported for wheat bran extracted with different solvents like acetone and ethanol (2.7-15.2 µmol Trolox/g bran) (Zhou & Yu, 2004) what indicates that SC-CO_2 used under certain conditions of pressure and temperature could be a good solvent for the extraction of antioxidants from wheat bran. Moore et al. (2006) reported higher ABTS scavenging capacity (16.2 to 21.5 µmol Trolox equivalents/g bran) for 20 hard winter wheat varieties grown at two locations extracted with 50% acetone. Antioxidant properties of wheat bran are highly affected by genotype and environment (Moore et al., 2006; Yu & Zhou, 2004; Zhou et al., 2004) thus, comparison of the antioxidant capacity of our extracts with values reported in the literature is limited.

According to the values obtained in this work, the wheat bran extract obtained by SFE might have an important potential as antioxidant; one gram of oil obtained at 55 MPa and 95 °C presents an antioxidant capacity (410 µmol Trolox equivalents) which is between that reported for 100 mL of orange juice (249 ± 3 µmol Trolox equivalents) and that

reported for 100 mL of tea infusion (631 ± 8 µmol Trolox equivalents) (Saura-Calixto & Goñi, 2006).

Table 2.4. Average fatty acid profile of wheat bran oil obtained by SFE for the runs R2-R10

Fatty acid	R2-R10 (mg/g oil)	SC-CO_2 [a] (mg/g oil)	SC-CO_2 [b] (mg/g oil)
Caprylic acid (C8:0)	0.3 ± 0.2		
Capric acid (C10:0)	0.13 ± 0.02		
Lauric acid (C12:0)	0.3 ± 0.1		
Myristic acid (C14:0)	1.00 ± 0.09		
Pentadecanoic acid (C15:0)	0.82 ± 0.06		
Palmitic acid (C16:0)	134 ± 3	139 ± 3	157 ± 8
Palmitoleic acid (C16:1 n-7)	1.02 ± 0.07	1.1 ± 0.1	
Heptadecanoic acid (C17:0)	0.87 ± 0.07		
Heptadecenoic acid (C17:1 n-7)	0.4 ± 0.2		
Steraric acid (C18:0)	10.0 ± 0.3	7.6 ± 0.7	37 ± 6
Oleic acid (C18:1 n-9)	128 ± 5	192 ± 5	159 ± 14
Vaccenic acid (C18:1 n-7)	6.1 ± 0.1		
Linoleic acid (C18:2 n-6)	457 ± 17	366 ± 8	337 ± 43
γ- linolenic acid (C18:3 n-6)	0.20 ± 0.06		
α- linolenic acid (C18:3 n-3)	40 ± 2	10.4 ± 0.7	
Arachidic acid (C20:0)	1.80 ± 0.08		
Gondoic acid (C20:1 n-9)	4.70 ± 0.06		
Eicosadienoic acid (C20:2 n-6)	0.79 ± 0.05		
Behenic acid (C22:0)	1.4 ± 0.3		
Docosadienoic acid (C22:2 n-6)	0.13 ± 0.07		
Lignoceric acid (C24:0)	1.56 ± 0.06		
Nervonic acid (C24:1 n-9)	0.31 ± 0.09		
Saturated fatty acids	152 ± 4	147 ± 4	194 ± 14
Monounsaturated fatty acids	141 ± 6	193 ± 5	159 ± 14
Polyunsaturated fatty acids	498 ± 19	376 ± 9	337 ± 43
Total fatty acids	791 ± 29	716 ± 18	690 ± 71

[a] Durante et al. (2012); [b]Athukorala et al. (2010)

3.3.4 Effect of process variables on oil fatty acids content and profile

The fatty acid analysis of the oils showed that there was no significant difference in the total amount of fatty acids in the oils obtained under the different extraction pressures and temperatures evaluated in this work. The average fatty acid content for runs R2-R10 and standard deviation are given in Table 2.4 (791 ± 29 mg of fatty acids/g oil). Also the fatty acid profile was similar in all the oils (see Table 2.4) being linoleic acid the major component (around 58% of the total fatty acids) followed by palmitic and oleic acids (around 16-17%). Table 2.4 also includes the fatty acid profile described in the literature for wheat bran oil obtained by SC-CO_2 (Athukorala et al., 2010; Durante et al., 2012). These authors have not evaluated the total profile, but just the major FA components what may explain the differences found. Also the different wheat varieties may highly affect the fatty acid profile.

3.4. Fractionation of wheat bran oil by using two-stage supercritical fluid extraction

Some authors reported the possibility of concentrating the AR components by performing SFE in two stages (Athukorala et al., 2010; Francisco et al., 2005a), the second one involving the use of cosolvents. In this work we have tried to achieve the same objective but instead of using cosolvents, we have performed a two-stage extraction (R11) using different extraction temperature and pressure, but pure CO_2. In the first stage, the extraction conditions chosen were those that had previously shown to provide the lowest ratio between the amount of AR and the amount of fatty acids, which were the lowest pressure and temperature used in this work. The second extraction stage was carried out under those conditions that had shown to provide the highest AR/fatty acids ratio, which were the highest pressure and temperature used in this work. The total extraction time was 120 minutes, 60 minutes for each stage. Table 2.5 shows the extraction yield and characterization of the corresponding extracts for the first and second extraction stages (Fraction 1 and Fraction 2 respectively). The extraction yield was higher in the first stage while the extract obtained during the second extraction stage was more concentrated in AR and polyphenols, and therefore with a higher antioxidant capacity. Their composition also differed in the amount of fatty acids; around 75% of the first fraction was fatty acids while in the second extraction step only the 40% corresponds to fatty acids. The aspect of the second fraction (Figure 2.5) suggested that it was concentrated in waxes and other lipids which could increase its viscosity resulting in an extract difficult to work with. Similar

fatty acid and AR profiles as those described above for wheat bran oils has been found for the two fractions obtained from the two stage extraction.

Table 2.5. Extraction yield and characterization of wheat bran oil obtained by two stage SFE

		Fraction 1	Fraction 2
Extraction pressure (MPa)		25 ± 1	55 ± 2
Extraction temperature (°C)		40 ± 1	83 ± 1
Extraction yield (g oil/100 g dry bran)		2.1 ± 0.5	1.1 ± 0.2
AR (mg/g oil)	Colorimetric method	35 ± 5	117 ± 20
	HPLC method	75 ± 9	234 ± 17
TPI (abs units/g oil)		752 ± 15	1667 ± 263
ABTS (mmol Trolox/g oil)		0.31 ± 0.03	0.58 ± 0.01
Fatty acids (mg/g oil)		751 ± 46	401 ± 42

Data are given as mean values ± SD; number of replicates n=3.

Figure 2.6. Examples of extracts obtained in the two-stage SFE: first stage (left) and second stage (right)

4. CONCLUSIONS

Wheat bran oil could be obtained by SFE, using pure CO_2 as solvent, with yields similar to those obtained using conventional organic solvents. The extraction yield presented crossover behavior and as a consequence, the influence of extraction temperature was

higher the higher the extraction pressure. Similar trends were found for the extraction of some bioactive compounds found in wheat bran, such as AR and other phenolic compounds. As a result, the highest values of extraction pressure and temperature used in this work (55 MPa and 95 °C) provided the bran oil with the highest antioxidant capacity.

Supercritical fluid fractionation allowed us to obtain wheat bran oil enriched with AR and other phenols and with higher antioxidant activity.

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Chapter 3

Characterization of wheat bran oil and evolution during storage

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Bioactive compounds and other quality parameters of wheat bran oil extracted with supercritical carbon dioxide

(sent for publication in Food Chemistry)

ABSTRACT

Wheat bran oil extracted with supercritical carbon dioxide has been analyzed for determining its bioactive compounds composition: a high content of valuable compounds such as, alkylresorcinols (47 mg/g), α-linolenic acid (37 mg/g), steryl ferulates (18 mg/g), tocopherols (7 mg/g) and phenolic compounds (25 ppm) has been found. Fresh oil quality evaluation showed a low level of hydroperoxides and hexanal and a remarkable antioxidant activity; however, a significant level of acidity was also found. Evolution of the wheat bran oil quality and composition during storage, at 21 °C and darkness, showed a good stability, with a slow decrease in oil quality after 90 days of storage: alkylresorcinols and tocopherols contents were reduced by 13% and 20%, respectively after 155 days due to their protection effect against oxidation. The results obtained indicate an attractive potential of wheat bran oil as a source of bioactive compounds for industrial and nutraceutical applications.

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1. INTRODUCTION

The addition of antioxidants is the most widely used strategy for reducing oxidation, and the consequent loss of quality in food products. The food industry has been always interested in lipophilic antioxidants, and the most commonly used have been some synthetic ones, as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). However, the often negative effects of these compounds on health and the increasing demand of natural additives by the consumers highlight the necessity of new lipophilic antioxidants that could control oxidation during processing and storage (Bramley et al., 2000). Furthermore, natural antioxidants are also demanded by the food industry for inhibiting the enzymes that produce browning and hyperpigmentation, which occur in plants and animals by the synthesis of melanin and other brown pigments, conducted by tyrosinase and other polyphenoloxidases (PPO) enzymes (Chen et al., 2005). In this sense, a wide variety of natural substances with a high phenolic content has been studied, to determine their capacity as tyrosinase inhibitors and their potential to replace the most commonly used sulphiting agents, which are known to produce allergic reactions, among others effects on human health (Nirmal & Benjakul, 2011).

Wheat bran is an important source of bioactive compounds, which are related to the health-protective mechanisms of whole-grain cereals (Fardet, 2010). Some of these bioactive compounds, such as alkylresorcinols (AR) and tocopherols, and to a lesser extent phenolic compounds, can be extracted by supercritical fluid extraction processes (Reverchon & Marco, 2006). This has been also shown on the results obtained in the Chapter 1 and 2 of the present work (Rebolleda et al., 2014; Rebolleda et al., 2013). However, wheat bran oil extracted with supercritical carbon dioxide (SC-CO_2) has been poorly characterized, and it is known that the solvent system used in the extraction process influences the oil composition and quality (Zhou & Yu, 2004); therefore, new studies on the levels of the different bioactive compounds and the antioxidant activity of supercritical extracted wheat bran oil are necessary in order to evaluate its potential uses in the food industry.

The aim of this chapter was to evaluate the bioactive compounds (alkylresorcinols, steryl ferulates, tocopherols and phenolic compounds) contained in wheat bran oil extracted with SC-CO_2. Furthermore, usual oil quality parameters and antioxidant capacity were also evaluated. Finally, the evolution of oil composition and quality during 155 days of storage, at 21 ± 1 ºC and darkness, was assessed.

2. MATERIALS AND METHODS

2.1. Raw material

Oil was extracted from wheat bran (*Triticum aestivum* L.) by supercritical fluid extraction (SFE) as previously reported (Chapter 2). Extraction conditions were 25.0 ± 0.1 MPa, 40 ± 2 °C and 8 ± 1 kg CO_2/h. Co-extracted water was removed by centrifugation at 12857*g* during 30 minutes. Conditions were selected to avoid working with emulsified oils.

2.2. Chemicals

Fatty acid methyl esters, AR (C15, C17, C19 and C25), phenolic (ferulic, vanillic and syringic acids, vanillin and *p*-OH-benzaldehyde) and tocopherol standards, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picryhydrazyl) and TPTZ (2,4,6-tri(2-pyridyl)-*s*-triazine), were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Syringic aldehyde was supplied by Extra synthese (Genay, France), methyl tricosanoate by Larodan (Malmö, Sweden) and $K_2O_8S_2$, $FeCl_3$ and $FeSO_4$ were purchased from Panreac (Barcelona, Spain).

2.3. Analytical methods

A full description of the analytical methods used in this chapter can be found in the Appendix B.

2.3.1 Determination of wheat bran oil bioactive compounds

Fatty acid content and profile

The fatty acid methyl esters were firstly prepared and then analyzed by gas chromatography (Agilent Technologies, Santa Clara, CA, USA) (AOAC, 1995). Split injection (50:1) and a flame ionization detector (FID), both at 250 °C, were used. The separation was carried out with helium (1.8 mL/min) as carrier gas in a fused silica capillary column (Omegawax TM-320, 30 m×0.32 mm i.d.). The column temperature was programmed starting at a constant temperature of 180 °C for 20 min, heated to 200 °C at 1 °C/min, held at 200 °C for 1 min, heated again to 220 °C at 5 °C/min and finally held at 220 °C for 20 min. Fatty acid methyl esters were identified and quantified using response factors obtained with their chromatographic standards and methyl tricosanoate as internal standard.

Alkylresorcinol content and profile

Alkylresorcinols were determined by HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA) according to a previously reported method (Rebolleda et al., 2013). The column used was a Kromasil C18-5 (250 x 4.6 mm) operated at 25 °C. The mobile phase was methanol (A) and water (B) and the following gradient was used: 2% B to 0% B in 10 min. The total run time was 50 min. The injection volume was 100 µL of methanolic solutions of wheat bran oil (10 mg/mL). All AR were monitored at 280 nm at a flow rate of 0.6 mL/min.

Steryl ferulate content and profile

Steryl ferulates were analyzed by HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA) according to a previously reported method, with some modifications (Mishra et al., 2012). Separation was carried out in a Zorbax XDB C18 column (150 x 4.6 mm, 5 µm) using isocratic elution with acetonitrile/methanol/isopropanol (50:40:10). Methanolic solutions of wheat bran oil (10 mg/mL) were injected (30 µL). Steryl ferulates were monitored at 330 nm, at a flow rate of 1 mL/min, and identified using a standard mixture of steryl ferulates and literature data (Hakala et al., 2002).

Tocopherol content and profile

Solid phase extraction (SPE) followed by HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA) was used for the determination of tocopherols in wheat bran oil according to a previously published methodology (Rebolleda et al., 2012). In a first step, tocopherols were extracted in silica cartridges (1000 mg/6 mL, Sep- Pak®, Waters, Spain) that were previously conditioned (5 mL *n*-hexane), charged with 1 mL of wheat bran oil solution (0.1 g/mL *n*-hexane) and equilibrated (5 mL *n*-hexane). Elution was performed with 5 mL of *n*-hexane–diethylether (99:1, v/v) and 50 mL of *n*-hexane–diethylether (99:2, v/v). The collected fraction was evaporated under reduced pressure at 45 °C and the dry residue obtained was dissolved in 1.5 mL of *n*-hexane. In a second step, 50 µL of this solution were injected in a HPLC. The column used was an ACE 5 silica column (250 mm × 4.6 mm) and the mobile phase was hexane and 2- propanol (99:1) at a flow rate of 1 mL/min during 15 minutes. Tocopherols were identified and quantified at 296 nm using calibration lines of the corresponding standard compounds.

Phenolic compounds content and profile

Phenolic compounds were extracted from wheat bran oil previous to their analysis by HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA). 2.0 g of oil were extracted with 2

mL of methanol (x2) by vortex agitation during 2 minutes. The methanol extracts were centrifuged at 3214*g* during 30 minutes and the supernatant was evaporated under *vacuum* at 40 °C. The dry residue was suspended in 2 mL of water: methanol (80:20), filtered (20 µm) and analyzed by a HPLC-DAD system according to the method previously reported by Pérez-Magariño et al. (2009). Chromatographic separation was performed in a Spherisorb ODS2-3µm column (4.6 x 250 mm) at a flow rate of 0.6 mL/min with (A) water/acetic acid (98:2) and (B) water/acetonitrile/acetic acid (78:20:2) and the following linear gradient: from 0 to 25% solvent B in 25 min, from 25 to 70% B in 35 min, from 70 to 100% B in 40 min and then isocratic for 20 min. Diode array detection was performed from 200 to 400 nm. The injection volume was 200 µL. The phenolic compounds analyzed were identified by comparing their retention times and UV-vis spectra with their respective standard according to previously published data (Pérez-Magariño et al., 2009). Quantification was performed by means of calibration lines obtained with the corresponding standard compound.

2.3.2 Determination of wheat bran oil quality parameters

Acidity value (AV)

A modification of the AOCS Ca 5a-40 (AOCS, 1990) method was used to evaluate the oil acidity by automatic titration with potassium hydroxide solution (Metrohm 905 Titrando, Herisau, Switzerland) using a pH electrode (Solvotrode, Metrohm, Herisau, Switzerland). Results were given as percentage of oleic acid.

Peroxide value (PV)

Peroxide value was determined potentiometrically according to a modification of the AOCS Cd 8-53 method (AOCS, 1990) by titration with sodium thiosulfate using an automatic titrator (Metrohm 905 Titrando, Herisau, Switzerland) equipped with a platinum electrode (Combined LL Pt-ring electrode, Metrohm, Herisau, Switzerland). Results were expressed in oxygen milliequivalents per kg of oil.

Hexanal content

Hexanal concentration was analyzed by GC–MS after solid phase dynamic extraction (SPDE) of the sample headspace (HS) using the method reported by Corcuera-Tecedor (2013). A coated SPDE-syringe with a non-polar 90% polydimethylsiloxane and 10% activated carbon sorbent (Chromtech, Idstein, Germany) was used in the HS-SPDE autosampler (CTC CombiPal autosampler, CTC Analytics,

Switzerland). A pre-equilibration step of 1 min at 70 °C was carried out. The coated needle was connected to a 2.5 mL gastight syringe and 50 extraction cycles of 1000 µL each, at a speed of 40 µL/s, were carried out. For the compounds desorption and injection, 500 µL of helium were pulled into the SPDE-syringe during 30 s and then pumped into the GC inlet at 15 µL/s. GC-MS analyses were performed using a gas chromatograph (Agilent Technologies 6890N, Network GC system) coupled to a mass selective spectrophotometer detector (Agilent Technologies, model 5973 inert). Separation was carried out in a capillary column (Carbowax 20M, 60 m×0.32 mm i.d.) with helium at a constant flow of 1.0 mL/min. The injector temperature was 250 °C and splitless injection mode was used. The initial oven temperature was 40 °C and it was increased to 240 °C at 3 °C/min. The mass spectrophotometer was set in electron-impact (EI) mode at 70 eV with a voltage multiplier of 1835 V.

2.3.3 Determination of wheat bran oil antioxidant capacity

ABTS assay

The total antioxidant activity of wheat bran oil was evaluated by the ABTS assay, which is applicable for both lipophilic and hydrophilic antioxidants. The radical $ABTS^{\bullet+}$ was generated by mixing 7 mM solution of ABTS in water with 2.45 mM $K_2O_8S_2$ (1:1) and held in darkness during 16 h (Rivero-Pérez et al., 2007). The $ABTS^{\bullet+}$ antioxidant reaction mixture contained 20 µL of wheat bran oil diluted in ethanol (5 mg/mL) and 980 µL of radical $ABTS^{\bullet+}$. Absorbance was measured at 734 nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan) after 20 minutes of reaction. Trolox was used as antioxidant standard.

FRAP assay

The reductive power of the wheat bran oil was evaluated by the FRAP assay (Benzie & Strain, 1996). Reaction takes place by mixing 30 µL of an ethanol solution of oil (5 mg/mL) and 970 µL of FRAP reagent. The FRAP reagent was prepared with 25 mL of 0.3 M sodium acetate buffer solution at pH 3.6, 2.5 mL of 10 mM TPTZ (tripyridyl-S-triazine), 2.5 mL of $FeCl_3$ (20 mM), and 3 mL of milli-Q water. The reaction was carried out at 37 °C during 30 minutes and the absorbance was measured at 593 nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan). $FeSO_4$ was used for calibration and the reductive power of the oil was expressed as µmol Fe (II).

DPPH assay

Free radical scavenging capacity of wheat bran oil was evaluated using 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) (Brand-Williams et al., 1995). 20 µL of wheat bran oil diluted in ethanol (5 mg/mL) were mixed with 980 µL of $DPPH^{\bullet}$ solution (50.7 µM) and the absorbance at 517 nm was measured (Hitachi U-2000 spectrophotometer, Tokyo, Japan) after 60 minutes of reaction at ambient temperature and darkness. Methanolic solutions of known Trolox concentrations were used for calibration.

2.3.4 Evolution of wheat bran oil composition and quality during storage

Wheat bran oil was stored at 21 ± 1 °C during 155 days,in darkness. The following parameters: AR and tocopherol content and profile, AV, PV, hexanal content and antioxidant capacity by the ABTS assay, were monitored over that time.

2.4. Statistical analysis

All the determinations were conducted in triplicate and results were expressed as mean ± standard deviation (SD). Differences between data means were compared by least significant differences (LSD) calculated using STATGRAPHICS Centurion XVI.I.

3. RESULTS AND DISCUSSION

Supercritical fluid technology was previously studied in Chapters 1 and 2 (Rebolleda et al., 2014; Rebolleda et al., 2013). Now, a wide evaluation of composition and quality of the wheat bran oil extracted under selected process conditions is presented.

3.1. Bioactive compounds in wheat bran oil

A large variety of bioactive compounds, such as alkylresorcinols (around 50 mg/g oil), tocopherols (around 7 mg/g oil), steryl ferulates (18.2 mg/g oil), phenolic compounds (around 25 ppm) and fatty acids (712 mg/g oil) were found in wheat bran oil extracted with SC-CO_2 (Table 3.1).

The fatty acid profile revealed that most of the fatty acids were polyunsaturated fatty acids (PUFA), around 63%, with a low amount of saturated fatty acids (around 18%). Linoleic acid (LA, C18:2ω6) was the major PUFA detected (around 58% of total fatty acids), and significant quantities of α-linolenic acid (ALA, C18:3ω3) were also quantified. Both compounds are essential PUFA, precursors of the omega-6 and omega-3 families, respectively, and therefore, very important in the human diet. Its large PUFA content

makes wheat bran oil to be considered of higher quality than some of the most commonly used oils, which usually have very low levels of PUFA (e.g. palm oil) and often show very low levels of ALA (e.g. sunflower, sesame, and palm oils) (Gunstone, 2002).

Alkylresorcinols have been described to have a wide range of biological activities such as antibacterial, antifungical, anticancer and enzyme inhibitor activities, among others (Bartlomiej et al., 2012). The intensity of these activities is different for each AR homologue (Parikka et al., 2006), which is probably due to the different length of the alkyl chain in the AR molecules. Information on the AR profile could be useful for decision making on its utilities. The AR profile of wheat bran oil extracted with SC-CO_2 (Table 3.1) is similar to that previously reported for wheat bran (Kulawinek et al., 2008), being C19 and C21 homologues the major ones (around 30% and 48%, respectively).

Steryl ferulates, which are esters of ferulic acid with sterols, have been widely described for rice bran oil, where they are known as γ-oryzanol. Health benefits associated with γ-oryzanol include hypocholesterolaemic–hypolipidaemic and anti-inflammatory activities (Lerma-García et al., 2009). Steryl ferulates are also considered to be potential antioxidants because of the hydrogen-donating ability of the phenolic group of ferulic acid (Nyström et al., 2005). The occurrence of ferulic acid esters of sterols in wheat grain and bran have been previously reported (Hakala et al., 2002; Kumar & Krishna, 2013; Nurmi et al., 2012; Nyström et al., 2007) but, to our knowledge, this work is the first one that describes the presence of steryl ferulates in a SC-CO_2 wheat bran extracted oil.

Significant levels of steryl ferulates were found in wheat bran oil extracted with SC-CO_2 (Table 3.1) being higher than those described for hexane extracted oils (Kumar & Krishna, 2013). Similar results have been reported for rice bran, where the γ-oryzanol yield was higher in supercritical extracted oil than in rice bran oil extracted with conventional solvents (Lerma-García et al., 2009). The steryl ferulate profile of SC-CO_2 extracted wheat bran oil (Table 3.1) was similar to that reported in acetone extracts of wheat bran (Nurmi et al., 2012) corresponding 54% to campestanyl + sitosteryl ferulates, 32% to sitostanyl ferulate and 13% campesteryl ferulate. Wheat bran steryl ferulates have shown good antioxidant activity even more than rice bran steryl ferulates (Nyström et al., 2005) which are mainly composed of 24-methylencycloartanyl and cycloartenyl ferulates (Hakala et al., 2002).

Table 3.1 . Bioactive compounds evaluated in wheat bran oil extracted with SC-CO_2

Families and compounds	Individual amounts and totals
Fatty acids (mg/g oil)	*712 ± 20*
Palmitic acid (C16:0)	118 ± 2
Steraric acid (C18:0)	7.9 ± 0.1
Oleic acid (C18:1)	114 ± 3
Linoleic acid (C18:2)	410 ± 10
α- linolenic acid (C18:3)	37.3 ± 0.8
Others	24.6 ± 4
Alkylresorcinols (mg/g oil)	*46.9 ± 0.8*
C15- AR	0.52 ± 0.01
C17- AR	3.3 ± 0.2
C19-AR	14.3 ± 0.3
C21- AR	22.4 ± 0.2
C23- AR	4.40 ± 0.03
C25- AR	1.97 ± 0.06
Steryl ferulates (mg/g oil wrt γ-oryzanol)	*18 ± 1*
Campesteryl ferulate	2.4 ± 0.1
Sitosteryl ferulate + campestanyl ferulate	9.9 ± 0.5
Sitostanyl ferulate	5.9 ± 0.4
Tocopherols (mg/g oil)	*6.8 ± 0.1*
α- T	3.84 ± 0.01
β- T	0.20 ± 0.01
γ-T	2.67 ± 0.08
δ-T	0.09 ± 0.01
Phenolic compounds (ppm)	*25 ± 2*
Vainillin	13.8 ± 0.1
Vanillic acid	3.5 ± 0.5
Syringic aldehyde	3.4 ± 0.8
Ferulic acid	1.8 ± 0.3
Syringic acid	1.6 ± 0.4
p-OH-benzaldehyde	0.7 ± 0.1

Values are mean ± standard deviation of three replicates (n=3). wrt with respect to

Tocopherol content of wheat bran oils extracted by supercritical fluid extraction has been scarcely reported. The mean tocopherol content of the wheat bran oil object of this study was 6.8 ± 0.1 mg/g, which is a much higher value than the described for other vegetable oils such as crude soybean oil and refined rice bran oil (Gunstone, 2002), and even for wheat bran oil also obtained by SFE but from *Triticum durum* variety (4.3 ± 0.7 mg/g oil) (Durante et al., 2012). It has been described that tocopherol composition differs among the different wheat varieties (Okarter et al., 2010), which, together with the effect of the extraction conditions, could explain the quantitative differences of tocopherol content described in each paper.

To our knowledge, the four tocopherol isomers have been detected and quantified for the first time in supercritical wheat bran oil (Table 3.1). This was possible because a normal phase chromatographic method, which enables the separation of β- and γ-tocopherols, was used. When reverse-phase columns were used, this separation was not possible (Bramley et al., 2000). The main tocopherols of the wheat bran oil obtained in this work were α-tocopherol (57%) and γ-tocopherol (39%). The α-tocopherol proportion is similar to that described for wheat germ and cottonseed oils, and the γ-tocopherol proportion is higher than that reported for sunflower and olive oils (Lampi et al., 2002). These results could be of interest to the food industry because it has been described that α-tocopherol presents high biological activity and γ-tocopherol has been reported to be the most effective tocopherol isomer to inhibit the oxidation of fats and oils (Bramley et al., 2000). Nevertheless, in recent years, different results suggest that the antioxidant activity of each tocopherol homologue depends, among other factors, on the food system where they are evaluated (Seppanen et al., 2010).

Wheat bran has been described as a rich source of phenolic compounds (Fardet, 2010). However, due to the low solubility of these compounds in supercritical CO_2 (Beltrán et al., 2008), low phenol content was found in the wheat bran oil obtained in this work (Table 3.1). The total phenolic compounds content (25 ppm) was found to be significantly lower than that reported for phenolic rich oils such as olive oil, which polyphenol content usually varies between 100 and 300 ppm (Gunstone, 2002). Previous works evaluated the global polyphenol content of supercritical wheat bran oil; however individual phenolic compounds have not been previously evaluated in this type of oil. Some of the main phenolic acids reported in wheat bran are ferulic, vanillic and syringic acids (Mattila et al., 2005). The solubility of these acids in supercritical CO_2 is higher for vanillic acid than for ferulic acid, and this, higher than for syringic acid

(Beltrán et al., 2008). These solubility differences may explain the predominance of vanillic acid in the wheat bran oil as well as the high level of vanillin and the presence of other aldehydes so in general, aldehydes show higher solubility in SC-CO_2 than their corresponding acids. Moreover, the absence in the oil of other phenolic acids such as *p*-cumaric and caffeic acids, also reported in wheat bran (Mattila et al., 2005), could be due to their low solubility in supercritical CO_2 (Beltrán et al., 2008).

3.2. Evaluation of oil quality

Wheat bran oil quality was evaluated using some of the common parameters used for these purpose. Hydrolysis of triglycerides and lipid oxidation have been described as the main factors of deterioration of edible oils. Triglycerides hydrolysis releases free fatty acids (FFA) susceptible of oxidation. Lipid oxidation occurs in two steps, the first one is the primary oxidation which is correlated to the oxidation of FFA and gives unstable hydroperoxides that are susceptible of decomposition during the secondary oxidation, in which a complex mixture of volatile, non-volatile, and polymeric products is formed. Secondary oxidation products include aldehydes, ketones, alcohols, etc., being hexanal one of them. Therefore, Acidity Value (AV), which is related with the free fatty acid content of the oil and Peroxide Value (PV) and hexanal content which are respectively related to the primary and secondary oxidation products, were chosen to evaluate wheat bran oil quality.

The AV of the SFE wheat bran oil under study was 15.2 ± 0.2% oleic acid, which indicates a significant quantity of FFA in the wheat bran oil. One of the problems of the high content of FFA in oils is their high susceptibility to oxidation with the consequent decrease of the oil quality and stability. The high AV obtained is probably due to the oxidation of wheat bran during storage. It is well-known that wheat lipase activity is present mainly in the bran fraction (O'Connor et al., 1992) and bran triacylglicerols are substantial subtracts to lipases. Physical damages occurring during wheat milling enhance the contact between enzymes and substrates, favoring the hydrolysis of triglycerides. Furthermore, the humidity of bran (around 11%) is enough to allow lipases action. Therefore, minimization of this type of oxidation should be avoided improving wheat bran storage conditions. Also, wheat bran oil AV could be reduced by performing SFE with on line fractionation of the extract, or else though a refining process.

The PV of the wheat bran oil obtained in this work indicated a low level of hydroperoxides (2.4 ± 0.1 meq/kg). The absence of oxygen during SFE has been correlated with lower PV

of SFE oils compared with the oils obtained by other extraction techniques (Khattab & Zeitoun, 2013). In contrast, Jung et al. (2012) reported higher PV (> 20 meq O_2/kg) but also lower AV (around 2.5 mg KOH/g). Both results seem to agree. High PV indicates strong oxidative degradation of free fatty acids, and consequently the levels of FFA decrease as much as PV increases. The low PV of the oil obtained in this work indicates a low oxidative degradation of the FFA, which remain intact giving the high AV detected. The drying and grinding steps used by Jung et al. (2012) could explain the intense oxidation detected in the oil that they studied. Such intense oxidation could also be related to the lower levels of antioxidants found in oil when dry bran was used in SFE than when wheat bran was not dry as it has been previously reported in Chapter 2 (Rebolleda et al., 2014).

The hexanal content is an important parameter to evaluate the oxidative deterioration of linoleic acid-containing oils and it has been related to the perception of rancidity in sensorial oil evaluation (Matthäus et al., 2010). The hexanal content of wheat bran oil just after SC-CO_2 extraction was 0.21 ± 0.03 ppb which is far below the odor and flavor threshold values reported for hexanal in oil which are 31 and 150 ppb, respectively (Leffingwell & Associates, 1989).

3.3. Evaluation of antioxidant activity

The system used for the evaluation of the antioxidant activity may influence the results and that is the reason why the use of two or more methodologies is highly recommended (Yu & Zhou, 2004). The antioxidant capacity of the wheat bran oil obtained in this work was evaluated by using two radical systems, ABTS and DPPH methods, and also the redox method FRAP (Table 3.2). The antioxidant activity evaluated by the DPPH method was higher than that reported by Durante et al. (2012) (1.90 µmol Trolox/g oil) for supercritical extracted wheat bran oil. This result may be explained considering the lower levels of tocopherols, strong antioxidants, detected in the oil obtained by the cited authors. Similarly, the antioxidant capacity evaluated by the ABTS method (270 µmol Trolox/g) was higher than that reported for other vegetable oils such as olive and sunflower oils (12.8 and 2.4 µmol Trolox/mL, respectively) (Saura-Calixto & Goñi, 2006). These differences could be explained considering the different antioxidant composition of the different oils. The oil under study showed higher levels of tocopherols than those reported for olive (0.1-0.3 mg/g) and crude sunflower oils (0.6-0.7 mg/g) (Gunstone, 2002). Furthermore, alkylresorcinols and steryl ferulates, which are also strong antioxidants, are not present in olive and sunflower oils.

Table 3.2. Antioxidant capacity of wheat bran oil extracted with SC-CO_2, evaluated by different methods

Antioxidant method	Antioxidant mechanism	Standard compound	Units	Value
DPPH	SET	Trolox	µmol Trolox/g	26 ± 2
FRAP	SET	$FeSO_4$	µmol Fe (II)/g	228 ± 12
ABTS	SET/HAT	Trolox	µmol Trolox/g	270 ± 6

SET: single electron transfer. HAT: hydrogen atom transfer

3.4. Evolution of wheat bran oil during storage

Quality oils should be stable, and usually oil stability is related to lipid oxidation. For that reason, parameters correlated with lipid oxidation were evaluated, at different time intervals, on wheat bran oil stored at 21 °C and darkness. AR and tocopherol contents, antioxidant activity measured by ABTS, AV, PV and hexanal content were the parameters chosen to monitor the stability of the wheat bran oil under study.

Regarding the content of bioactive compounds of the oil, results showed that levels of total AR remain about constant until the second month of storage, but showed statistically significant differences from 90 days of storage (Figure 3.1). Global losses of AR around 13% were obtained after 155 days of storage. C17 and C19 AR showed the higher degradation ratios, around 18 and 16%, respectively, while C23 decreased around 7% and C15 around 4%. No relationship between the chain length and the losses of each AR was found.

Qualitative and quantitative losses of tocopherols were also observed (Figure 3.2), and they were more intense for γ-tocopherol (33%) than for α-tocopherol (11%) at the end of the storage time. These results are similar to those described for olive oils stored at 20 °C and darkness (Fadda et al., 2012). These authors found reductions ranging from 6 to 9% for α-tocopherol and from 35 to 44% for β+γ-tocopherol, after 180 days of storage. These results agree with previous ones (Bramley et al., 2000), which indicated that fatty acid composition and storage conditions might influence the evolution of each tocopherol isomer during storage.

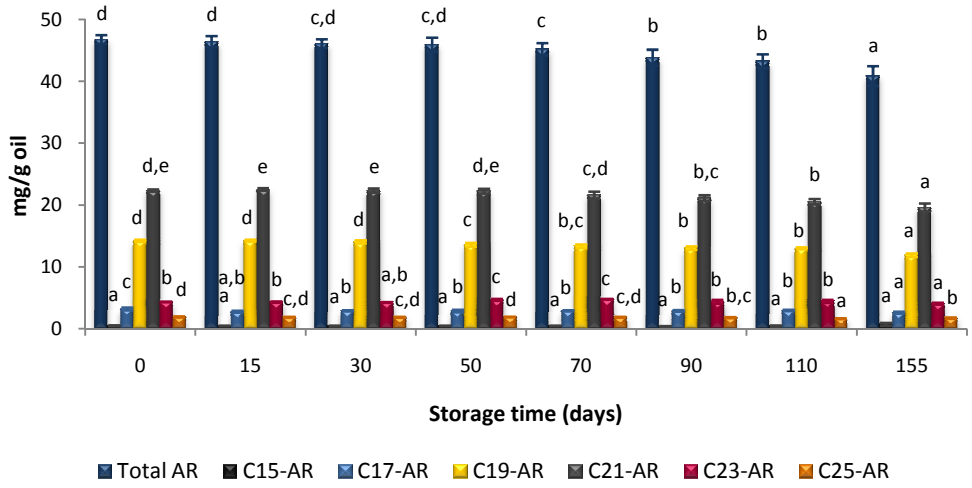

Figure 3.1. Alkylresorcinol content and profile of wheat bran oil during storage at 21 ± 1°C and darkness. Different letters within the same compound indicate significant mean differences according to LSD test at $p < 0.05$

The higher decrease of γ-tocopherol could be correlated with its protective effect against oxidation of oils. This is supported by the AV results, which values remained about constant until the second month of storage (Figure 3.3). Obviously, tocopherols were not able to totally inhibit the oxidation of FFA and for that reason PV increased from the first month of storage. As more peroxide was generated, more intense was the oxidation of FFA, more tocopherols were degraded, and more peroxides were accumulated, until secondary oxidation began to be predominant after the third month of storage, when the hexanal content started growing (Figure 3.3). Despite this process, hexanal content remained below the odor and flavor threshold value of hexanal (Leffingwell & Associates, 1989) and also far below the limit reported for the perception of rancidity in other oils (1 ppm) (Matthäus et al., 2010). From the third month of storage, when oxidation is large, other antioxidants present in bran oil such as AR decrease due to their lipid oxidation inhibition action (Figure 3.1).

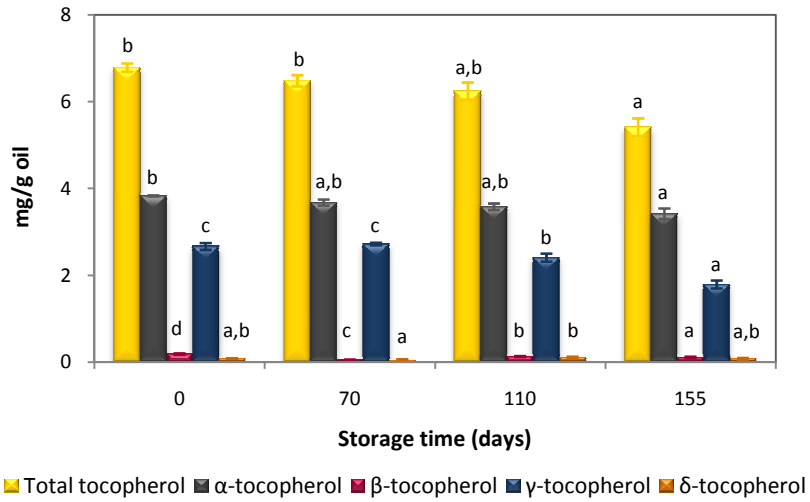

Figure 3.2. Tocopherol content and profile of wheat bran oil during storage at 21 ± 1ºC and darkness. Different letters within the same compound indicate significant mean differences according to LSD test at $p < 0.05$

The PV ratio of 155 days stored and fresh oil was calculated in order to compare the PV evolution with that described for other oils stored under similar conditions,. The obtained value (14.9) is much higher than that described for lavender oil (> 6.5) but lower than that described for pine oil (> 16.3) (Turek & Stintzing, 2012).

The evolution of the antioxidant capacity of the wheat bran oil under study during storage was monitored by the ABTS assay. Results showed a relative stability during the first month, and a slow decrease afterwards. Statistically significant differences between fresh and stored oil were found starting from 50 days of storage. These results are well correlated with the progressive loss of antioxidant compounds, and it is specially associated with the loss of α-tocopherol, which decreased slower than γ-tocopherol. In any case, after the storage period evaluated, wheat bran oil still retained an important antioxidant activity, being much higher than the antioxidant activity reported for different essential oils (Tawaha et al., 2007) and also for other vegetable oils such as olive and sunflower oils (Saura-Calixto & Goñi, 2006).

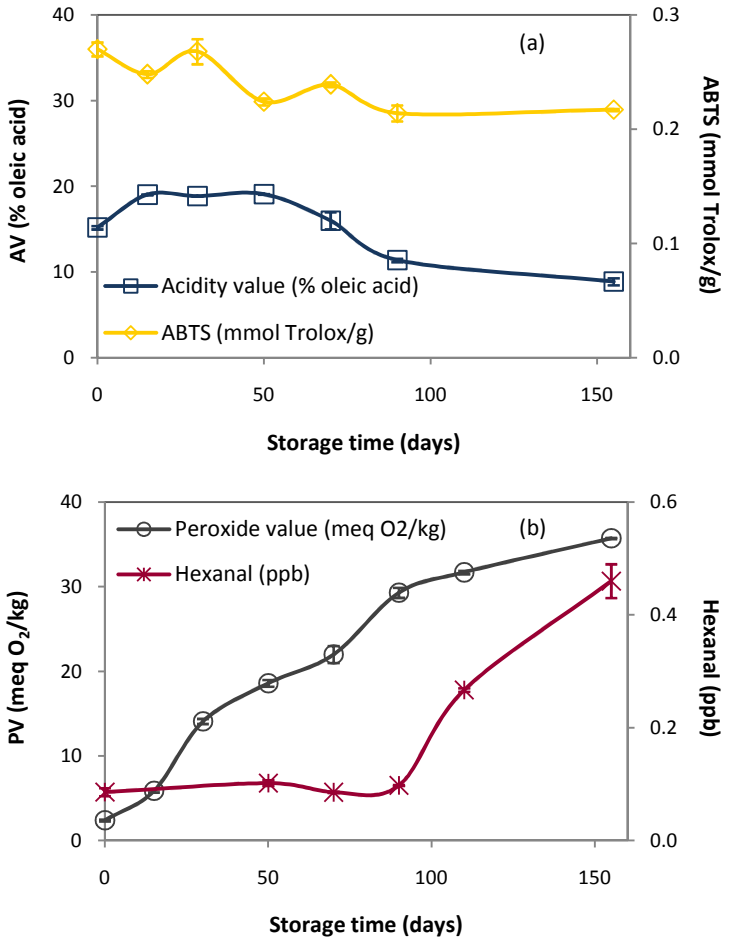

Figure 3.3. Evolution of: (a) acidity value (AV) and antioxidant activity (ABTS values) and (b) peroxide value (PV) and hexanal content, of wheat bran oil extracted with supercritical CO_2, measured during a storage period of 155 days at 21 ± 1 °C and darkness

4. CONCLUSIONS

Wheat bran oil extracted with supercritical CO_2 showed a high content of valuable bioactive compounds such as alkylresorcinols, steryl ferulates and tocopherols with a small amount of other phenolic compounds. A low level of oxidation, with low hydroperoxides and hexanal content, was found in the fresh wheat bran oil, suggesting a high stability of this oil. However, the moderate level of free fatty acids indicate that the storage conditions of wheat bran should be well controlled in order to avoid or, at least, minimize the oxidation process, otherwise the obtained oil have to undergo refining process.

Evolution of oil composition and quality parameters indicated that wheat bran oil obtained by SFE is a stable product with very interesting properties to be used in food industry. The high antioxidant activity and bioactive compounds content, and the good stability at room temperature storage (around 21 ºC), even having an initial high AV, evidence the potential uses as natural antioxidant and also as a possible functional ingredient.

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Chapter 4

Formulation and characterization of wheat bran oil-in-water nanoemulsions

Rebolleda, S., Benito, J. M., Sanz, M. T., Beltrán, S., Escudero, I., & González-Sanjosé, M. L., Formulation and characterization of wheat bran oil-in-water nanoemulsions. *Food Chemistry DOI: 10.1016/j.foodchem.2014.06.097*

ABSTRACT

Wheat bran oil has been reported to have an important content of bioactive compounds such as tocopherols, alkylresorcinols and other phenolic compounds; however, its poor solubility in water systems restricts its applications in the food industry. This chapter is focused on the formulation of oil-in-water (O/W) nanoemulsions of wheat bran oil in order to improve the bioaccessibility of its active compounds. The influence of oil concentration, surfactant type and concentration, and emulsification method, on the droplet size and stability of the nanoemulsions was investigated. Response surface methodology was used to optimize the conditions for preparing stable nanoemulsions with the minimum droplet size. The optimal nanoemulsion was obtained when 1% of wheat bran oil and 7.3% of a surfactant mixture of Span 80 (37.4%) and Tween 80 (62.6%) were emulsified in water by high intensity ultrasonication for 50 s after pre-emulsification with a high speed blender during 5 min. The optimal nanoemulsion showed good stability along time which make it suitable for use in food applications.

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1. INTRODUCTION

There has been growing interest in the utilization of natural antioxidants in the food, beverage and pharmaceutical industries due to the increasing consumer´s demand for substituting synthetic compounds by natural substances. Several vegetal by-products have been proved to be a good source of functional ingredients (Herrero et al., 2006). One of these by-products is wheat bran, which has been successfully extracted using supercritical fluid extraction (SFE) giving rise to extracts that have shown an important content on tocopherols, alkylresorcinols and other phenolic compounds, which provide them with a good antioxidant activity (Chapters 1-3) (Rebolleda et al., 2014; Rebolleda et al., 2013).

Due to its lipophilic character, wheat bran oil must be formulated before it can be used for aqueous-based matrix applications. The high stability and low turbidity of nanoemulsions (10-200 nm) make them suitable to incorporate lipophilic active ingredients in aqueous-based food and beverages (McClements, 2011; Yang et al., 2012). Furthermore, nanoemulsions have been described as drug delivery systems and as adequate media to overcome instability and to enhance the bioavailability of nutraceuticals (Huang et al., 2010; Karadag et al., 2013; Peshkovsky et al., 2013; Tadros et al., 2004). For all these reasons, nanoemulsions have an increasing interest in the food, cosmetic and pharmaceutical industries.

Different factors, such as the type of oil and surfactant and process conditions influence the physicochemical properties of nanoemulsions (Einhorn-Stoll et al., 2002; McClements, 2011). The composition of the dispersed oily phase considerably influences the emulsion quality because of the different densities, viscosities and surface-active ingredients of the different type of oils (Einhorn-Stoll et al., 2002). Some of the oily phases that have been used for the formation of nanoemulsions are limonene oil (Jafari et al., 2007; Li & Chiang, 2012), sunflower oil (Leong et al., 2009), and medium chain triglycerides (Yang et al., 2012; Yuan et al., 2008). These oily phases are in most cases used to dissolve bioactive compounds; however, wheat bran oil obtained by SFE already contains highly bioactive compounds, hence, in this chapter, wheat bran oil will be directly emulsified.

The specific objective of the present chapter was to optimize some process variables, such as oil concentration, surfactant type and concentration, and emulsification method, to obtain stable wheat bran oil-in-water (O/W) nanoemulsions with the minimum possible droplet size. Response surface methodology (RSM) was applied to detect the optimal

conditions. Additionally, emulsion stability along time of the optimal nanoemulsion was evaluated.

2. MATERIALS AND METHODS

2.1. Materials

Oil phase: wheat bran oil was obtained by SFE in a semi-pilot plant at 25.0 ± 0.1 MPa, 40 ± 2 °C and 8 ± 1 kg CO_2/h. Co-extracted water was separated from wheat bran oil by centrifugation at 12857*g* during 30 minutes. Wheat bran oil was stored at -20 °C until the emulsification experiments were performed. Its alkylresorcinols and tocopherols content were found to be around 47 and 7 mg/g of oil, respectively. A wider characterization of the wheat bran oil used in this work has been reported in Chapter 3.

*Surfactants: s*everal food grade surfactants have been selected in order to achieve the stabilization of O/W nanoemulsions. Table 4.1 compiles the different surfactants and mixtures of surfactants used, together with their HLB (hydrophilic-lipophilic balance) number. Tween 80 (polyoxyethylene (20) sorbitan monooleate) and Span 80 (sorbitan monooleate) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA), Tween 20 (polyoxyethylene (20) sorbitan monolaurate) by Panreac (Barcelona, Spain) and DATEM (diacetyl tartaric acid ester of mono- and diglycerides) by EPSA (Valencia, Spain).

Water phase: milli-Q water (Millipore, Billerica, MA, USA) was used to prepare all emulsions.

2.2. Equipment and procedure

A vortex (VWR, Radnor, PA, USA), a high speed blender (Miccra D9 equipped with a DS-5/K-1 rotor-stator, ART Labortechnik, Mülhein, Germany), an ultrasonic bath (Selecta Ultrasounds H, Barcelona, Spain) and a high intensity ultrasonic processor (Sonics VCX 500, Newtown, CT, USA) were the apparatus used for preparing the emulsions. The high intensity ultrasonic processor (500 W, 20 kHz) was used with a titanium alloy micro tip probe of 3 mm diameter, at 20% of amplitude and in pulses of 5 seconds (5 s ultrasound and 5 s pause) to avoid the heating of the sample.

To prepare an emulsion, wheat bran oil and surfactant were mixed before water milli-Q was added. Quantities of each emulsion ingredient were measured using an analytical balance (Sartorius, accurate ± 0.0001). The characterization of the emulsions was performed an hour after emulsification to avoid any creaming or coalescence effect.

2.3. Nanoemulsions characterization

Droplet size distribution, mean droplet diameter and polydispersity index (PDI) of samples were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS apparatus (Malvern Instruments Ltd., UK). The apparatus is equipped with a He-Ne laser emitting at 633 nm and with a 4.0 mW power source. The instrument uses a backscattering configuration where detection is done at a scattering angle of 173°. Samples were first diluted 1:100 to avoid multiple scattering effects and then 2 mL samples were poured into DTS0012 square disposable polystyrene cuvettes. Measurements were performed at 20 °C. The hydrodynamic diameter was calculated using the Stokes-Einstein equation with the assumption that the particles were monodisperse spheres.

Zeta potential was measured using the afore mentioned Zetasizer Nano ZS apparatus. The measurement was conducted for each diluted sample at 20 °C using DTS1061 disposable folded capillary cells. The Zeta potential, ζ, was calculated from oil droplet electrophoretic mobility measurements in an applied electric field using the Smoluchowski approximation.

The refractive index of the dispersed phase, wheat bran oil, was experimentally determined (Milton Roy abbe-type refractometer, Ivyland, PA, USA) resulting to be 1.476 at 25 °C.

The pH of the nanoemulsions was measured by means of a glass pH electrode (Crison, Barcelona, Spain).

Turbidity analysis of the formulated emulsions was carried out by measuring the absorbance of undiluted samples at 600 nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan) (Ghosh et al., 2013).

2.4. Evaluation of nanoemulsions stability

Stability of wheat bran oil in water nanoemulsions was measured in terms of their droplet growth ratio. Since emulsions tend to aggregate during storage, the droplet size of the emulsions obtained in this work was measured at 15 and 30 days at the bottom of the cell containing them. Two different storage conditions were evaluated: 4 °C and darkness and 20 °C and lightness.

Additionally, optical characterization of creaming stability was made for the optimal nanoemulsion using a Turbiscan Lab Expert equipment (Formulaction Co., L'Union, France) by static multiple light scattering (MLS), sending a light beam from an electroluminescent diode (λ = 880 nm) through a cylindrical glass cell containing the sample. The

nanoemulsion sample without dilution was placed in a cylindrical glass cell and two synchronous optical sensors received the light transmitted through the sample (180° from the incident light) and the light backscattered by the droplets in the sample (45° from the incident light). The optical reading head scans the height of the sample in the cell (about 40 mm), by acquiring transmission and backscattering data every 40 µm. Transmitted and backscattered light were monitored as a function of time and cell height for 60 days at 25 °C (Allende et al., 2008).

2.5. Experimental design

The effect of two of the factors under study, surfactant type and the emulsification procedure, on emulsion formation was firstly studied. Then, response surface methodology (RSM) and central composite design (CCD) were used to study the effect of oil and emulsifier concentration and emulsification time, on the droplet size of the nanoemulsions.

The experiments performed to select the surfactant are presented in Table 4.1. Emulsions of wheat bran oil (1% w/w) with different emulsifiers (1% w/w) were obtained working with the high speed blender at 29000 rpm during 5 minutes. Each experiment was replicated twice.

The emulsification method was selected by preparing different emulsions of wheat bran oil (1% w/w) using the surfactant (1% w/w) selected in the previous assays. The emulsification procedures used in each experiment are shown in Table 4.2. Each experiment was replicated twice.

After selecting the surfactant type and emulsification method, response surface methodology (RSM) was used to study the effect of oil concentration (X_1: 1-10% w/w), emulsifier concentration (X_2: 1-10% w/w) and ultrasonication time (X_3: 50-300 s) on the droplet size of the nanoemulsions (Y). A central composite design (CCD) with three levels of each independent variable (Table 4.3) was used. The model generated 17 experimental settings with three replicates in the central point. The design was carried out by duplicate.

A low degree polynomial equation (second-order one) was used to express predicted responses (Y) as a function of the independent variables under study (X_1, X_2 and X_3). The model equation is as follows:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{11} X_1^2 + \alpha_{22} X_2^2 + \alpha_{33} X_3^2 + \alpha_{12} X_1 X_2 + \alpha_{13} X_1 X_3 + \alpha_{23} X_2 X_3 \quad \text{(Eq. 4.1)}$$

where Y represents the response variable (droplet size), α_o is a constant, and α_i, α_{ii}, α_{ij} are the linear, quadratic and interactive coefficients, respectively. The significance of each estimated regression coefficient was assessed through values of the statistic parameters, F and p (*probability*). The experimental design and data analysis were performed using STATGRAPHICS Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA).

2.6. Statistical analysis

Analysis of variance (ANOVA) and LSD test were applied to detect the effect factor and the statistically significant differences among values, respectively.

Data analysis was performed using STATGRAPHICS Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

In this section, the influence of surfactant type and emulsification procedure on droplet size is analyzed in the first place. Then, the details of the RSM used for obtaining the values of the rest of the process variables considered (oil and surfactant concentration and high intensity ultrasonication time) leading to the nanoemulsion with the minimum droplet size are presented. Finally, the characterization of the optimal nanoemulsions is presented.

3.1. Influence of surfactant type on nanoemulsion droplet size

The type of surfactant or mixture of surfactants used for formulating the different emulsions prepared for this study is presented in Table 4.1 together with their HLB number. Table 4.1 also shows the droplet size and PDI of the emulsions formulated with the different surfactant systems.

ANOVA analysis showed surfactant factor effect and all the analytical values were statistically different among them. The minimum droplet size (84.6 ± 1.3 nm) and the narrowest particle size distribution (PDI = 0.257 ± 0.009) were obtained when the mixture of Span 80 (37.4%) and Tween 80 (62.6%), with a HLB value of 11, was used. This agrees with empirical observations that suggest that minimum droplet size and maximum emulsion stability is obtained for O/W emulsions when using surfactants with a HLB number within the range 10-12 (McClements, 2005).

According to the results obtained in this section, the experiments presented in the next sections were carried out using the above mentioned surfactant mixture.

Table 4.1. Surfactants used for the emulsification of wheat bran oil, their HLB numbers and mean droplet size and polydispersity index (PDI) of the emulsions obtained

Surfactant	HLB number	Droplet size (nm)	PDI
Tween 20	16.7	200.0 ± 8.1^{d}	0.352 ± 0.016
Tween 80	15.0	109.9 ± 6.6^{b}	0.370 ± 0.009
DATEM	8.0	393.2 ± 8.0^{e}	0.485 ± 0.011
Span 80:Tween 80 (37.4:62.6)	11.0	84.6 ± 1.3^{a}	0.257 ± 0.009
Span 80:Tween 80 (75:25)	7.0	171.1 ± 3.9^{c}	0.280 ± 0.012

Values with different letters in each column are significantly different (LSD test, $p < 0.05$)

3.2. Influence of the emulsification method on nanoemulsion droplet size

The energy needed for the emulsification process can be provided by mechanical agitation, (e.g.: stirring, high shear mixing), high-pressure homogenization or high power ultrasound (Huang et al., 2010; Peshkovsky et al., 2013). Although high-pressure homogenization is widely used, ultrasonic methods have several advantages, such as lower-cost equipment, smaller footprint and easier cleaning and servicing (Peshkovsky et al., 2013).

Some assays were carried out in order to choose the emulsification method that provided an emulsion with a small droplet size (Table 4.2). ANOVA analysis showed emulsification procedure factor effect. Although the methods using high speed blender happened to bring the smallest droplet size, samples exhibited visual creaming instability after a few hours of storage. In contrast, nanoemulsions obtained with the high intensity ultrasonic processor showed a slightly larger droplet size but visual instability was not observed after the same storage period. Similar results were obtained by Einhorn-Stoll et al. (2002), who observed a rapid destabilization of emulsions prepared by a single step with Ultra-turrax. The ultrasonic bath was considered not suitable for the formation of these nanoemulsions because, after 10 min, it did not produce the emulsification of the entire oil phase.

Table 4.2. Equipment and conditions used for the emulsification of wheat bran oil and mean droplet size and polydispersity index (PDI) of the emulsions obtained

Pre-emulsification step		Emulsification step		Droplet size (nm)	PDI
Method	Time (min)	Method	Time (min)		
None		High speed blender 25000 rpm	5.0	95.6 ± 1.6^{c}	0.234 ± 0.008^{c}
None		High speed blender 29000 rpm	5.0	77.0 ± 1.1^{b}	0.225 ± 0.013^{c}
None		High speed blender 35000 rpm	5.0	66.3 ± 0.9^{a}	0.187 ± 0.005^{b}
None		Ultrasonic bath	10	-	-
None		High intensity ultrasonic processor	2.5	136.4 ± 1.3^{e}	0.121 ± 0.010^{a}
Vortex	1	High intensity ultrasonic processor	2.5	133.5 ± 1.3^{e}	0.139 ± 0.009^{a}
High speed blender 29000 rpm	5	High intensity ultrasonic processor	2.5	111.0 ± 0.8^{d}	0.132 ± 0.008^{a}

Values with different letters in each column are significantly different (LSD test, $p < 0.05$)

According to these results, emulsification by high intensity ultrasonication was selected as the emulsification procedure for the next experiments. However, ultrasonication requires a large amount of energy when used directly to emulsify two separate phases; therefore, a pre-emulsification stage might be preferred to first prepare a coarse emulsion (Canselier et al., 2002). In this context, the possibility of adding such a pre-emulsification step using a vortex or a high speed blender was evaluated. Table 4.2 shows that the smallest droplet size (111.0 ± 0.8 nm) was obtained when this pre-emulsification was performed using the high speed blender. According to all the results obtained in this section, emulsification by high intensity ultrasonication preceded by a pre-emulsification with a high speed blender (29000 rpm, 5 min) was the method selected for carrying out the experiments presented in the next sections.

3.3. Influence of oil and surfactant concentration and ultrasonication time on nanoemulsions droplet size. Search of the optimal conditions by RSM

The results on the RSM used to optimize the formulation of wheat bran nanoemulsions with the minimum droplet size, taking into account the process variables oil and surfactant concentration and ultrasonication time, are firstly presented. Additionally, stability of the different nanoemulsions is discussed.

3.3.1. Model fitting

The droplet size of the wheat bran nanoemulsions obtained in the experiments corresponding to the CCD design is given in Table 4.3. The experimental data were fitted to a quadratic polynomial equation, which was able to correctly predict the droplet size of the emulsions. The model obtained was robust, showed no lack of fit (*p* value was higher than 0.05, Table 4.4), a high value of the correlation coefficient (R^2=0.986) and the distribution of the residuals was normal. All the coefficients of the quadratic polynomial model (Eq. 4.1) were statistically significant ($p< 0.05$) except for the interactive coefficient α_{13} (Table 4.4). F values indicate that, for the range of surfactant concentration studied, the oil content and ultrasonication time had stronger incidence on the droplet size of the emulsions than the surfactant content. F values also indicate that the interaction with the highest incidence was the one occurring between the quantity of surfactant and the ultrasonication time.

3.3.2. Response surface analysis

In order to study the effect of the independent variables on the droplet size, surface response and contour plots of the quadratic polynomial model were generated by varying two of the independent variables within the experimental range while holding the third one constant at the central point. Fig. 4.1a was generated by varying the oil and surfactant content in the emulsion while holding constant the ultrasonication time at 175 s. It shows that, at constant oil content, an increase of surfactant content between 1 and 7% (w/w) results in a decrease of droplet size of the emulsion, while a surfactant content higher than 7% results in a droplet size increase. This could be explained by the role of the surfactant in the emulsion, since its concentration determines the total droplet surface area, the diffusion rate and the adsorption phenomena of the surfactant onto the newly formed droplets. Excessive surfactant content might lead to a lower diffusion rate of surfactants which can result in the coalescence of the emulsion droplets (Li & Chiang, 2012).

Table 4.3. Matrix of the central composite design (CCD) and experimental data obtained for the response variable (Y)

Run	Independent variables			Response variable
	Wheat bran oil concentration (X_1, % w/w)	Surfactant concentration (X_2, % w/w)	Ultrasonication time (X_3, s)	Droplet size (Y, nm) (mean ± SD)
1	5.5	5.5	175	155.7 ± 9.1
2	1.0	1.0	50	81.5 ± 4.2
3	10.0	1.0	50	199.0 ± 0.7
4	1.0	10.0	50	50.7 ± 2.0
5	10.0	10.0	50	138.1 ± 0.8
6	1.0	1.0	300	116.8 ± 6.2
7	10.0	1.0	300	226.1 ± 1.3
8	1.0	10.0	300	143.6 ± 1.8
9	5.5	5.5	175	154.3 ± 4.2
10	10.0	10.0	300	247.3 ± 20.4
11	1.0	5.5	175	92.1 ± 3.4
12	10.0	5.5	175	210.4 ± 15.4
13	5.5	1.0	175	184.8 ± 2.1
14	5.5	10.0	175	174.8 ± 1.2
15	5.5	5.5	50	105.2 ± 1.3
16	5.5	5.5	300	167.4 ± 0.4
17	5.5	5.5	175	149.5 ± 1.3

The effect of oil content and ultrasonication time on the droplet size at a fixed surfactant content of 5.5% can be observed in Fig. 4.1b. This Figure shows that the droplet size of the nanoemulsion increases both with ultrasonication time and oil content. Ultrasonication time is an important emulsification parameter, since it affects the adsorption rate of the surfactants to the droplet surface and the droplet size distribution (Li & Chiang, 2012). The

increase of the droplet size of the emulsion when the ultrasonication time is increased has been described in the literature and it is due to the over-processing of the emulsion (Fathi et al., 2012; Kentish et al., 2008; Li & Chiang, 2012). This effect makes necessary the optimization of the ultrasonic energy intensity input for the system under study (Chandrapala et al., 2012). The same effect of ultrasonication time and surfactant content on the droplet size of the emulsions, previously discussed, was also observed when holding constant the oil content (Fig.4.1c).

Table 4.4. Analyses of variance of the regression coefficients of the quadratic equation (Eq.4.1) for the droplet size of wheat bran oil nanoemulsions

Polynomial coefficient (PC)[a]	PC-value	*F*-value	*p*-value
α_0	58.9453		
α_1	16.1043	2384.05	0.0000
α_2	-17.6021	24.05	0.0080
α_3	0.552472	884.93	0.0000
α_{11}	-0.299374	8.17	0.0460
α_{22}	1.10803	111.87	0.0005
α_{33}	-0.00134799	98.58	0.0006
α_{12}	-0.220679	13.25	0.0220
α_{13}	0.00181111	0.69	0.4533
α_{23}	0.0310333	202.18	0.0001
Lack of fit		2.55	0.1883

[a] α_0 is a constant, α_i, α_{ii} and α_{ij} are the linear, quadratic and interactive coefficients of the quadratic polynomial equation, respectively

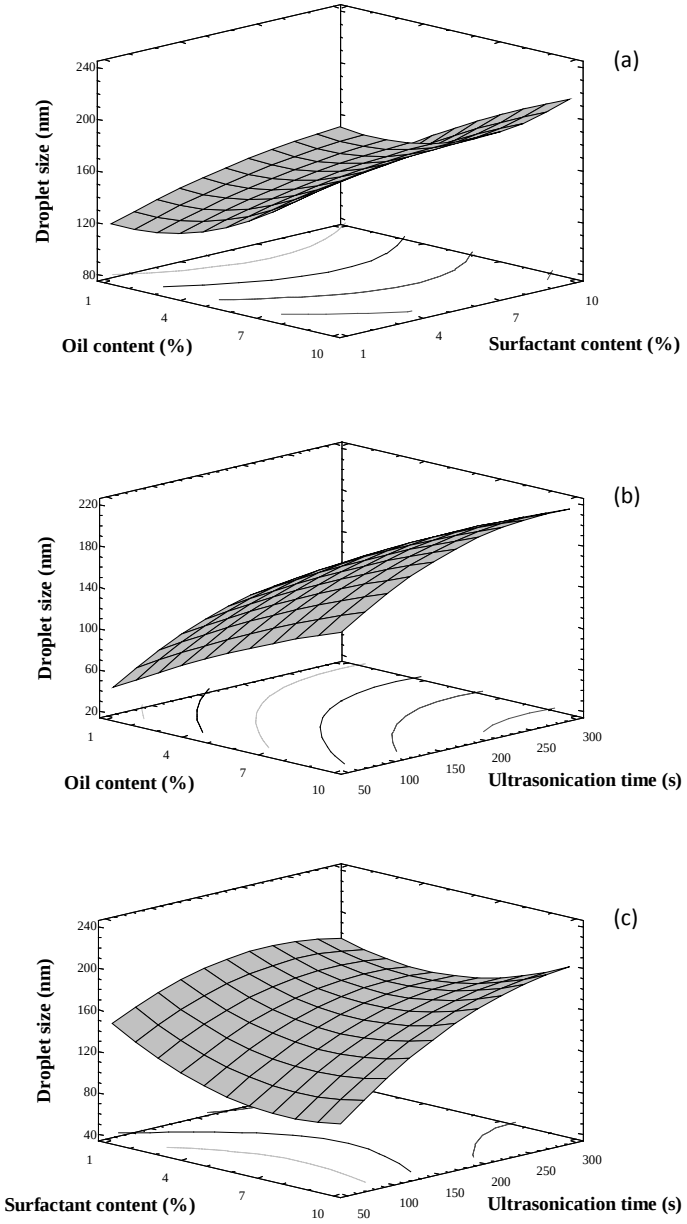

Figure 1.1. Response surface and contour plots of the combined effects of oil and surfactant content and ultrasonication time on the droplet size of the wheat bran nanoemulsions: (a) oil and surfactant content at an ultrasonication time of 175 s; (b) oil content and ultrasonication time at a surfactant content of 5.5%; (c) surfactant content and ultrasonication time at an oil content of 5.5%.

3.3.3. Stability of wheat bran oil nanoemulsions

Once the influence of the process variables on the droplet size has been evaluated, it is important to check if there are some important effects of these variables on emulsion stability, since this is one of the most important parameters for their application.

Zeta potential provides information on emulsion stability and is determined by measuring the velocity of charged droplets or colloids in an electrical potential field of known strength. Oil droplets in an O/W emulsion exhibit a net charge at the droplet surface. It is usually a negative charge, and as described by the Helmholtz theory of the electrical double layer, the negative charges are aligned or closely bound to the interface. These charges attract counterions from the bulk solution which give rise to a zone of opposite sign, forming an electrical double layer that causes oil droplets to repel one another. Hence, zeta potential is an indication of the repulsive forces between emulsion oil droplets, thus characterizes coalescence/flocculation capacity of emulsions and reflects its stability (Kumar et al., 2013). Large zeta potential values (positive or negative) indicate difficulty for coalescence of droplets and therefore high emulsion stability. The zeta potential of the emulsions corresponding to the CCD experiments varies from -30 to -40 mV, indicating good stability.

The stability of the emulsions was also evaluated in terms of their droplet size growth and appearance when they were stored during 15 and 30 days at lightness and ambient temperature and when they were stored at 4 ºC and darkness. There was little change in the droplet size of the emulsions during storage (data not shown) but significant changes in their appearance were detected in terms of sedimentation. Sedimentation is a reversible destabilization phenomena in emulsions while modification on the droplet size is an irreversible one (Abismaïl et al., 1999). Visual evaluation of the emulsions formulated in this work showed that sedimentation was higher in the emulsions with higher oil content. Emulsions stored at 4 ºC and darkness showed less sedimentation than those emulsions stored at ambient temperature and lightness when their visual appearance was evaluated.

3.3.4. Optimal conditions for preparing wheat bran oil nanoemulsions

The optimal conditions for the emulsification of the wheat bran oil used in this work would be those leading to a stable emulsion with the minimum droplet size. According to the RSM results, the minimum droplet size (39.4 nm) was predicted to be achieved by

combining 1.0% (w/w) wheat bran oil, 7.3% (w/w) surfactant and an ultrasonication time of 50 seconds.

It should be noted that the surfactant to oil ratio of the optimal wheat bran emulsion is relatively high what in practice is not desirable due to economic, sensorial and regulatory reasons (Qian & McClements, 2011). In any case, this is a minor problem when nanoemulsions are going to be applied as diluted forms, as in the case of this work, which objective is obtaining an emulsion with the minimum droplet size in order to improve the bioavailability of the wheat bran oil antioxidant compounds.

A confirmation of the results using the optimum conditions (1% oil, 7.3% surfactant and 50 s ultrasonication) was carried out by performing five replicates. The average droplet size obtained was of 39.9 ± 0.4 nm. The results showed that there was no significant difference ($p > 0.05$) between experimental and predicted values. The low PDI obtained (0.249 ± 0.012) indicates a narrow distribution of the droplet size.

3.4. Characterization of the optimal nanoemulsion

Besides determining the average droplet size and PDI of the optimal nanoemulsion some other parameters were evaluated for further characterization. The zeta potential was found to be -22 ± 2 mV and pH 4.9 ± 0.1. The low turbidity of the optimal nanoemulsion (600 nm absorbance = 0.36 ± 0.01) is related to their small droplet size which is below the detection limit of the human eye (around 50 nm) (Leong et al., 2009). This fact makes this nanoemulsion suitable for its incorporation into different systems without altering their visual quality. Also, the stability during storage of wheat bran oil optimal nanoemulsion was evaluated.

3.4.1. Stability along storage

There was no significant change in droplet size for the optimal nanoemulsions after 15 and 60 days of storage at 4 °C (Figure 4.2a), with no noticeable changes on visual emulsion stability. However, creaming stability measurements for 60 days at 25 °C using the Turbiscan Lab Expert apparatus (Figure 4.2b) showed that there was a slight backscattering increase along time for the middle zone of the measurement cell, which indicates an increase in droplet size caused by the coalescence of oil droplets. The formation of a sedimentation front at the bottom of the sample (about 3 mm of cell height) was also observed during the last days, indicating a tiny emulsion destabilization at the end of the storage period.

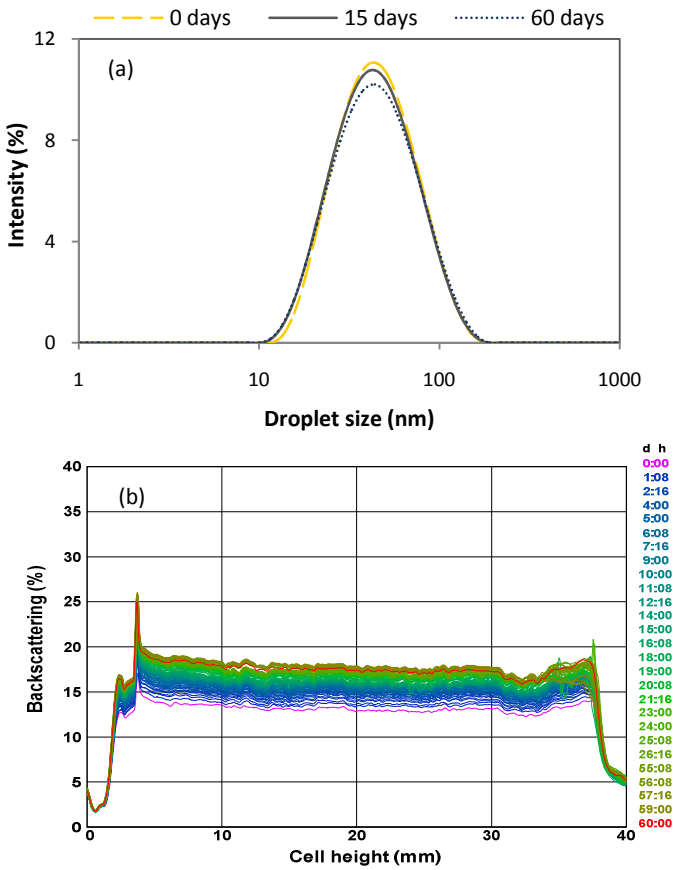

Figure 4.2. Optimal nanoemulsion prepared with 1% of wheat bran oil, 7.3% Span 80:Tween 80 (37.4:62.6) and 50 seconds of ultrasounds: (a) droplet size distribution after 0, 15 and 60 days of storage at 4 °C and darkness, (b) backscattering profiles during 60 days of storage at 25 °C and darkness.

4. CONCLUSIONS

Wheat bran oil can be successfully incorporated into water systems by the formulation of nanoemulsions. Optimization of process conditions by RSM showed that nanoemulsions with a droplet size of 40 nm can be obtained with a combination of high speed blender (29000 rpm- 5 min) and ultrasonic processor (50 seconds) using 1% of wheat bran oil and 7.3% of a surfactant mixture (Span 80 (37.4%) and Tween 80 (62.6%)). Nanoemulsions showed good stability when stored at 4 °C during 60 days and only a small destabilization was observed in the last days of the storage when stored at 25 °C during 60 days.

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Chapter 5

Evaluation of polyphenoloxidase inhibitory capacity of wheat bran oil nanoemulsions

ABSTRACT

The inhibitory capacity of wheat bran oil nanoemulsions on mushroom tyrosinase and prawn (*Marsupenaeus japonicus)* polyphenoloxidase has been evaluated. Two different wheat bran oils extracted with supercritical CO_2 under different extraction conditions were used for the formation of the nanoemulsions. AR and polyphenol content of the oils and antioxidant activity of the nanoemulsions were evaluated. Nanoemulsions formulated with the oil that showed the highest AR and polyphenol content, exhibited the highest antioxidant activity. The efficacy of the nanoemulsions for reducing the relative activity of mushroom tyrosinase and prawn polyphenoloxidase was in a dose dependent manner. The nanoemulsion with the highest antioxidant activity was found to be the most effective; inhibition of mushroom tyrosinase ranged from 41-67% when 0.5-2.5% of nanoemulsion was added in the reaction medium and inhibition of prawn polyphenoloxidase ranged from 8-86% when 8-40% of nanoemulsion was added.

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1. INTRODUCTION

Kuruma prawn *(Marsupenaeus japonicus)* is a commercially important penaeid which has been farmed in Japan since the late 1950s being introduced in a number of European countries (Italy, France, Spain and Portugal) for pond aquaculture in the 1980s (Quigley et al., 2013).

Prawns are a very perishable product, and post-mortem changes occur rapidly. Prawns generally have limited shelf life due to the formation of black spots (melanosis). Although the presence of these black spots seems to be harmless to consumers, it drastically reduces the market value of the product and its acceptability, leading to considerable financial losses. Also, microbial spoilage and lipid oxidation contribute to the unacceptability of prawn products (Nirmal & Benjakul, 2009b).

Melanosis is a process that is triggered by a biochemical mechanism consisting of oxidation of phenols to quinones by means of an enzymatic complex known as polyphenoloxidase (PPO). This is followed by non-enzymatic polymerization, giving rise to pigments of high molecular weight and very dark or black coloring (Montero et al., 2001).

Polyphenoloxidase also known as phenoloxidase, tyrosinase and phenolase is a copper oxidoreductase that catalyses two basic reactions, in the presence of molecular oxygen, the *o*-hydroxylation of monophenols to give *o*-diphenols (monophenoloxidase, EC 1.14.18.1) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenoloxidase, EC 1.10.3.1) (Junquera et al., 1992; Nirmal & Benjakul, 2012).

The black spots on prawns begin to be formed in the head and proceed down the prawn, forming black lines just under the shell that outlines the sections of the tails. Refrigeration or storage on ice slows down, but does not prevent this reaction because the PPO enzyme systems remain active under these conditions (Gokoglu & Yerlikaya, 2008).

Melanosis formation can be controlled by different actions such as decreasing the levels of enzyme substrates, reduction of *o*-quinones to diphenols, removal of oxygen, chelation of copper ion, which acts as cofactor of the PPO, and use of inhibitors.

Reducing agents are very effective in the control of browning. Sulphite derivatives are the most widely applied reagents for the control of melanosis in crustaceans, but these compounds are known to produce allergic reactions and serious disturbances in asthmatic subjects. Increased regulatory attention and heightened consumer awareness of the risks associated with sulphite added foods have created a need for a safe effective sulphite

alternative for use in foods (Gokoglu & Yerlikaya, 2008). Different physical methods that have been evaluated for the prevention of melanosis in prawns are the modified atmospheres which reduce the oxygen in the medium and the high pressure processing (Montero et al., 2001). Among the chemicals that inhibit melanosis, 4-hexylresocinol has become a synthetic effective alternative to sulphite derivates that has been approved in the EU for the prevention of browning (Directive 2006/52/CE). Hexylresorcinol is also used in USA, Australia and other countries for preventing browning in crustaceans, usually by immersion (Martínez-Alvarez et al., 2009; Montero et al., 2004).

The search of other natural antioxidants, especially of plant origin, has greatly increased in recent years (Gokoglu & Yerlikaya, 2008). Different plant extracts such as mango seed kernel extract (Maisuthisakul & Gordon, 2009) or black rice bran (Miyazawa et al., 2003) have shown inhibitory effect on commercial mushroom tyrosinase (EC1.14.18.1). Also, resorcinolic lipids isolated from *Anacardium occidentale* have been indicated as effective inhibitors of mushroom tyrosinase (Kubo et al., 1994; Zhuang et al., 2010).

The application of plant extracts for the inhibition of prawn melanosis has not been widely reported. Tea and grape seed extracts have been proved to be effective for delaying melanosis in prawn species (Gokoglu & Yerlikaya, 2008; Nirmal & Benjakul, 2011).

The high content in alkylresorcinols (AR) and other antioxidant compounds of supercritical fluid extracted wheat bran oil (Chapters 1-3) suggest that it may show inhibition capacity of PPO. It is therefore, the main objective of this work, the evaluation of the inhibitory capacity of wheat bran oil, formulated as nanoemulsion for its application, on both the mushroom tyrosinase and the PPO from the cephalothoraxes of prawns (*Marsupenaeus japonicus*).

2. MATERIALS AND METHODS

2.1. Chemicals and materials

AR standards (C15, C17, C19 and C25), Span 80 (sorbitan monooleate), Tween 80 (polyoxyethylene (20) sorbitan monooleate), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine), L-DOPA (3,4-dihydroxy-L-phenylalanine), mushroom tyrosinase (EC 1.14.18.1) and 4-hexylresorcinol were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). $K_2O_8S_2$, $FeCl_3$ and $FeSO_4$ were obtained from Panreac (Barcelona, Spain), Brij-35 was purchased

from Merck (Darmstadt, Germany) and sodium metabisulphite ($Na_2S_2O_5$) was supplied by VWR (Radnor, PA).

2.2. Extraction and characterization of wheat bran oil

2.2.1. Supercritical fluid extraction (SFE) of wheat bran oil

Wheat bran oil was extracted with supercritical CO_2 as it has been previously reported (Chapter 2). Two different oils, which differ in their content in antioxidant compounds, were obtained by using the following extraction conditions: oil A was extracted at 25 ± 1 MPa and 42 ± 2 °C and oil B was extracted at 50 ± 2MPa and 90 ± 3 °C.

2.2.2. Determination of the AR content of wheat bran oil

Wheat bran oil AR were evaluated by HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA) as it has been previously reported (Rebolleda et al., 2013). The column used was a Kromasil C18-5 (250 x 4.6 mm) operated at 25 °C. The mobile phase was methanol (A) and water (B) and the following gradient was used: 2% B to 0% B in 10 min. The total run time was 50 min. The injection volume was 100 µL of methanolic solutions of wheat bran oil (10 mg/mL). All AR were monitored at 280 nm at a flow rate of 0.6 mL/min.

2.2.3. Determination of total polyphenol index (TPI)

The TPI is based on the maximum absorbance that many phenolic compounds present at 280 nm. Ethanol solutions of wheat bran oil (5 mg/mL) were diluted with ethanol (1:10) and their absorbance at 280nm was measured (Hitachi U−2000 spectrophotometer, Tokyo, Japan).

2.3. Production and characterization of wheat bran oil nanoemulsions

2.3.1. Formation of wheat bran oil nanoemulsions

The supercritical extracted wheat bran oils were emulsified according to the following optimal conditions (Chapter 4): 1% of wheat bran oil was mixed with 7.3% of a surfactant mixture formed by Span 80 (37.4%) and Tween 80 (62.6%), before water milli-Q was added. Then, emulsification was carried out by using high speed blending at 29000 rpm during 5 minutes (Miccra D9ART Labortechnik, Mülhein, Germany) followed by ultrasonic processing at 20% of amplitude during 50 seconds (Sonics VCX 500, Newtown, CT, USA).

2.3.2. Evaluation of nanoemulsions antioxidant activity

The antioxidant properties of wheat bran oil nanoemulsions were evaluated by using the ABTS, DPPH and FRAP methods (Rebolleda et al., 2014; Rebolleda et al., 2013).

ABTS: The radical was produced by reaction of 7 mM solution of ABTS in water with 2.45 mM $K_2O_8S_2$ (1:1) during 16 h at room temperature and darkness (Rivero-Pérez et al., 2007). 20 µL of diluted nanoemulsion (1:4) were mixed with 980 µL of radical $ABTS^{\bullet+}$ previously diluted until obtaining 0.7 absorbance units at 734 nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan). The discoloration produced after 20 min of reaction was directly correlated with the antioxidant capacity of the products. Trolox was used as standard compound.

DPPH: 20 µL of nanoemulsion were mixed with 980 µL of $DPPH^{\bullet}$ solution (50.7 µM). The absorbance at 517 nm was measured after 60 minutes of reaction at ambient temperature and darkness (Hitachi U-2000 spectrophotometer, Tokyo Japan). The discoloration produced was directly correlated with the antioxidant capacity of the products. Trolox was used as standard compound.

FRAP: The FRAP reagent was prepared by mixing 25 mL of 0.3 M sodium acetate buffer solution at pH 3.6, 2.5mL of a 10 mM solution of TPTZ dissolved in HCl 40 mM, 2.5 mL of $FeCl_3$ (20 mM), and 3 mL of milli-Q water. 30 µL of nanoemulsions were mixed with 970 µL of FRAP reagent. The reaction was carried out at 37 ºC during 30 minutes and the absorbance was measured at 593nm (Hitachi U-2000 spectrophotometer, Tokyo, Japan). $FeSO_4$ was used for calibration. The reductive power of the nanoemulsions was expressed as µmol Fe (II).

2.4. Inhibition of enzyme activity by wheat bran oil nanoemulsions

2.4.1. Determination of mushroom tyrosinase inhibitory activity

The effect of the nanoemulsions on the *o*-diphenolase activity was monitored by the formation of dopachrome at 490 nm. The reaction medium (0.2 mL) contained 0.5 mM L-DOPA prepared in 100 mM phosphate buffer of pH 7; 0.1 mg/mL of mushroom tyrosinase also prepared in 100 mM phosphate buffer of pH 7; and different concentrations (0.5 to 2.5%, v/v) of the nanoemulsion. The absorbance at 490 nm was continuously monitored over a time period of 5 minutes (Labsystems Multiskan MS microplate reader). The initial reaction rate in the presence or absence of the

nanoemulsions was calculated from the slope of the reaction curve and the relative activity and inhibition of the nanoemulsions was calculated as follows:

$$\text{Relative activity } (\%) = \frac{Vi}{Vo} * 100 \quad \text{(Eq.5.1)}$$

$$\text{Inhibition } (\%) = 100\text{-relative activity} \quad \text{(Eq.5.2)}$$

where Vi and Vo are the initial reaction rates in the presence and absence of nanoemulsion, respectively.

2.4.2. Determination of PPO inhibitory activity

Preparation of PPO extract from prawn cephalothoraxes

Isolation of PPO from prawn cephalothoraxes was carried out according to the method reported by Nirmal and Benjakul (2009a). Frozen prawns, without any treatment, were kindly supplied by HASENOSA (Spain). Cephalothoraxes were separated, pooled and powderised by grinding with liquid nitrogen in a blender (Moulinex, Group SEB, Ecully, France). The powder (50 g) was mixed with 150 mL of extracting buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij-35). The mixture was stirred continuously at 4 °C during 30 minutes, and then centrifuged at 8000*g* for 30 minutes at 4 °C using a refrigerated centrifuge (Kontron, model T-124, Milano, Italy). Solid ammonium sulphate was added into the supernatant to obtain 40% saturation and the mixture was allowed to stand at 4 °C for 30 minutes. The precipitate was collected by centrifugation at 12500*g* at 4 °C for 30 min using a refrigerated centrifuge. The pellet obtained was dissolved in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2 and dialysed (Novagen D-Tube dyalizers, 12-14 kDa, Millipore Billerica, MA) against 15 vol of the same buffer at 4 °C with two changes of dialysis buffer. The insoluble materials were removed by centrifugation at 3000*g* at 4 °C for 30 minutes and the supernatant was used as ‘‘crude PPO extract”.

Inhibition PPO assay

The inhibitory effect of wheat bran oil nanoemulsions was evaluated according to a method previously reported (Nirmal & Benjakul, 2009a) with some modifications. The pH of nanoemulsions was previously adjusted to 6 with NaOH 0.1 N. First, different concentrations of nanoemulsions were mixed with 0.05 M phosphate buffer to a final volume of 1 mL, then 200 µL of crude PPO extract was added. To initiate the reaction 1.2 mL of pre-incubated 15 mM L-DOPA (45 °C) was added. Final concentrations in the

reaction medium (2.4 mL) were 8.33% (v/v) of crude PPO extract, 7.5 mM L-DOPA and variable amounts of inhibitor (nanoemulsions). Absorbance at 475 nm was monitored during 3 minutes (Hitachi U‐2000 spectrophotometer, Tokyo, Japan) and the relative activity and inhibition capacity were expressed according to Eq.5.1 and Eq.5.2, respectively.

With the aim of comparison, the commercial inhibitors sodium metabisulphite (2.5, 5 and 10 µg/mL reaction medium) and 4-hexylresorcinol (50, 100 and 230 µg/mL reaction medium) were also evaluated for the inhibition of the PPO. A theoretical yield of 67.4% of SO_2 was considered for sodium metabisulphite (Ough & Were, 2005).

2.5. Statistical analysis

All the determinations were conducted in triplicate and results were expressed as mean ± standard deviation (SD). Differences between data means were compared by least significant differences (LSD) calculated using STATGRAPHICS Centurion XVI.I.

3. RESULTS AND DISCUSSION

3.1. Characterization of wheat bran oils and their nanoemulsions

Two different wheat bran oils were extracted and formulated in nanoemulsions for their evaluation as inhibitors of PPO. The AR content and total polyphenol index (TPI) of the oils and the antioxidant activity of the nanoemulsions have been assessed (Table 5.1).

Table 5.1. Characterization of wheat bran oil and its nanoemulsions

		A	B
Extraction pressure (MPa)		25 ± 1	50 ± 2
Extraction temperature (°C)		42 ± 2	90 ± 3
Oil composition	AR content (mg/g)	43.3 ± 0.8	83 ± 1
	TPI (uds abs/g)	564 ± 14	871 ± 26
Nanoemulsion antioxidant activity	DPPH (mmol Trolox/L)	0.22 ± 0.01	0.26 ± 0.01
	FRAP (mmol Fe (II)/L)	0.57 ± 0.03	1.12 ± 0.04
	ABTS (mmol Trolox/L)	2.7 ± 0.1	3.3 ± 0.1

As it has been previously discussed (Chapter 1 and 2), the extraction conditions influence the AR and polyphenol content of the wheat bran oil and as a consequence, the antioxidant activity of the nanoemulsions that are formulated with those oils. Oil B, which was extracted at higher extraction pressure and temperature than oil A, showed higher AR and polyphenol content. According to the different composition of the oils, significant differences were found in the antioxidant activity of their nanoemulsions, being the antioxidant activity of nanoemulsion B higher than the antioxidant activity of nanoemulsion A, no matter the method used for its evaluation (Table 5.1). In the following sections, enzyme inhibition activities of both nanoemulsions have been evaluated and related to their antioxidant activity and oil composition.

3.2. Evaluation of the enzyme inhibitory capacity of wheat bran oil nanoemulsions

3.2.1. Effect of wheat bran oil nanoemulsions on the inhibition of mushroom tyrosinase

Tyrosinase catalyzes the oxidation of L-DOPA into dopachrome (diphenolase activity). Commercial mushroom tyrosinase was the enzyme chosen for performing some preliminary assays focused to determine the inhibitory capacity of wheat bran nanoemulsions.

Figure 5.1 shows the relative activity of mushroom tyrosinase for different concentrations of nanoemulsion A or B added to the reaction medium. The inhibitory capacity of the two nanoemulsions presented statistically significant differences at all the concentrations studied, being nanoemulsion B more active than A for the inhibition of mushroom tyrosinase under the assay conditions evaluated. The inhibitory capacity of nanoemulsion A was found to range from 31 to 54% when the nanoemulsion concentration in the reaction medium ranged from 0.5 to 2.5%, while the inhibitory capacity of nanoemulsion B ranged from 40 to 67% for the same nanoemulsion concentration range. Differences in the inhibition capacity could be related to the different composition and antioxidant capacity of the nanoemulsions. As oil B, used in the formulation of nanoemulsion B, showed a higher content in AR and polyphenols than oil A, the addition of the same concentration of nanoemulsion leads to a higher concentration of antioxidants in the reaction medium comparing when nanoemulsion A was added.

Other natural inhibitors of mushroom tyrosinase cited in the literature are cardol triene (pentadectrienylresorcinol) and kojic acid. Zhuang et al. (2010) studied the inhibitory kinetics of cardol triene (C15:3), which is a resorcinolic lipid isolated from cashew nut shell, on mushroom tyrosinase. These authors found that cardol triene was a powerful

inhibitor showing an IC_{50} value of 7.1 µg/mL, where IC_{50} is the inhibitor concentration necessary to reduce 50% the enzyme activity. For kojic acid, which is a fungal metabolite produced by *Aspergillus* and *Penicillium* species, around 50% of mushroom tyrosinase inhibition has been reported when 12 µg/mL were added to the reaction medium (Saruno et al., 1979). Due to the different experimental conditions only an approximate comparison with our results can be established; 58% of inhibition was achieved when 1.25% of nanoemulsion B was added , which corresponds to an AR concentration of 10.8 µg/mL of reaction medium (Figure 5.1).

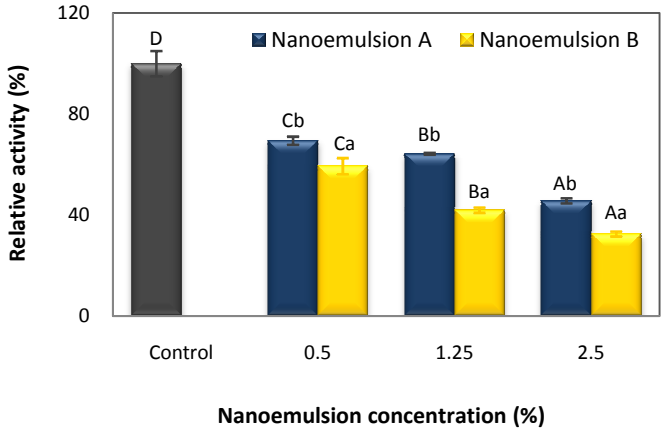

Figure 5.1. Effect of wheat bran nanoemulsions concentration on the inhibition of mushroom tyrosinase. Different capital letters within the same nanoemulsion and the control indicate statistically significant mean differences ($p < 0.05$). Different lower case letters within the same nanoemulsion concentration indicate statistically significant mean differences ($p < 0.05$)

3.2.2. Effect of wheat bran oil nanoemulsions on the inhibition of prawn PPO

The behavior of inhibitors in a model system cannot be extrapolated to a real system but it is useful as an approach to the mechanics of the inhibitor action and to explore the effectiveness of inhibitors (Montero et al., 2001).

The effect of the different concentrations of wheat bran oil nanoemulsions (A and B) on the inhibition of prawn PPO is shown in Figure 5.2. For both nanoemulsions, PPO relative activity was observed to decrease when the nanoemulsion concentration in the reaction medium increased ($p < 0.05$). Nanoemulsion A, with lower AR and polyphenol content, did not affect the PPO activity when added at 8% concentration, what seems to indicate that a

minimum concentration of antioxidants is necessary to achieve a significant reduction on the activity of the prawn enzyme.

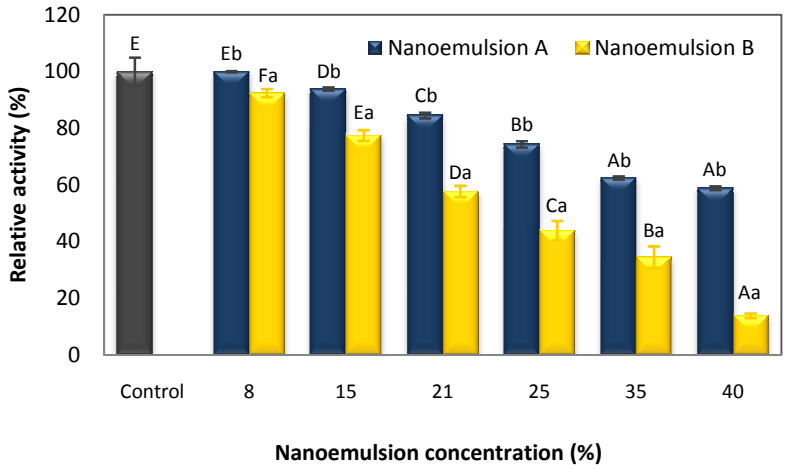

Figure 5.2. Effect of wheat bran nanoemulsions concentration on the inhibition of polyphenoloxidase from prawns. Different capital letters within the same nanoemulsion and control indicate significant mean differences ($p < 0.05$). Different lower case letters within the same nanoemulsion concentration indicate significant differences ($p < 0.05$)

The two nanoemulsions evaluated showed different effect on the PPO activity. Nanoemulsion A, showed an inhibitory capacity ranging from 0-41% for the different nanoemulsion concentrations evaluated (8-40%) while, nanoemulsion B, showed an inhibitory capacity ranging from 8-86% for the same nanoemulsion concentrations. The higher inhibitory capacity of nanoemulsion B could be related to the higher antioxidant capacity of this nanoemulsion comparing with nanoemulsion A. As previously mentioned, the AR and polyphenol content found in the oil used for preparing nanoemulsion B was higher than the content found in nanoemulsion A.

Although the comparison with other results reported in the literature is limited due to the different PPO characteristics of each prawn variety (Montero et al., 2001), a concentration-dependent inhibitory PPO capacity was also found for other natural extracts such as tea and mulberry extracts. An inhibitory capacity of 52-56% has been reported for green tea extract (0.17 mg/mL reaction medium) on PPO from white shrimp (Nirmal & Benjakul, 2011). For the nanoemulsions evaluated in this work, 56% of inhibition

was achieved when 25% of nanoemulsion B was used which corresponds to 2.44 mg oil/mL reaction medium.

A linear relationship was found between the AR concentration in the reaction medium and the inhibitory capacity on the prawn PPO (Figure 5.3). Results showed that a minimum concentration of AR was necessary to observed PPO inhibition as it has been previously mentioned. For the maximum AR concentration evaluated in this work (344 µg AR/mL), the PPO activity of the prawn extract was inhibited up to 86%. Nirmal and Benjakul (2009a) reported 40% inhibition on PPO activity of white shrimp extract when 1% ferulic acid was used which corresponds to 1.7 mg ferulic acid/mL reaction medium and it is around ten times higher than the AR concentration necessary to obtain the same inhibition (0.18 mg/mL).

It should be noted that AR are the main, but not the only antioxidants found in wheat bran oil (see Chapter 3) and therefore, the PPO inhibitory capacity of the nanoemulsions tested may be the result of the combined action of all the antioxidants present in the reaction medium.

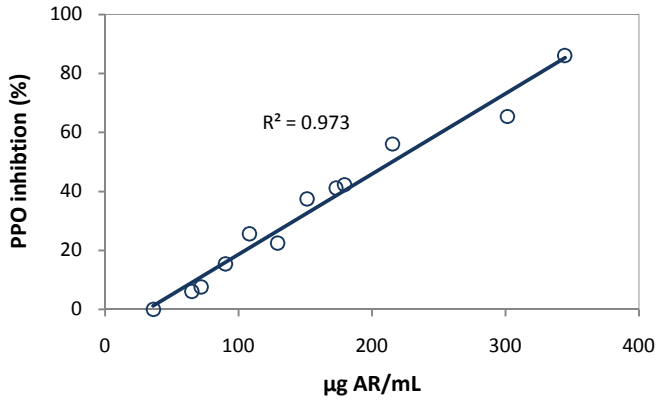

Figure 5.3. Relationship between AR concentration in the reaction medium and PPO inhibitory activity

In order to compare the inhibitory capacity of wheat bran oil nanoemulsions with other antibrowning agents usually employed in the food industry, the same assay was carried out with sulphite (as sodium metabisulphite) and hexylresorcinol. Table 5.2 shows the relative PPO activity obtained for the different concentrations of inhibitors employed together with the relative activity of three different concentrations of nanoemulsion B (25, 35 and 40%), which correspond with an AR concentration in the reaction medium of 215,

301 and 344 µg AR/mL. Sulphite was found to be a more effective inhibitor than 4-hexylresorcinol when evaluated in prawn PPO extract. Concentration of 4-hexyresorcinol was fifteen times higher than of sulphite to obtain the same inhibitory effect (Table 5.2). Montero et al. (2001) also reported that sulphite was more effective than 4-hexylresorcinol for the inhibition of prawn PPO. However, when shrimps were treated with these inhibitors, 4-hexylresorcinol was found to be more effective than sulphite for preventing the melanosis of the product (Guandalini et al., 1998; Martínez-Alvarez et al., 2009).

Table 5.2. Effect of sulphite, 4-hexylresorcinol and alkylresorcinols contained in wheat bran oil nanoemulsions on the relative activity of prawn polyphenoloxidase

Inhibitor	Concentration (µg/mL)	Relative activity (%)
Control	-	100 ± 8^{e}
Sulphite	1.7	43 ± 2^{d}
	3.4	14 ± 2^{b}
	6.7	4 ± 1^{a}
4-Hexylresorcinol	50	11.0 ± 0.1^{b}
	100	4.2 ± 0.3^{a}
	230	1.3 ± 0.1^{a}
AR	215	44 ± 4^{d}
	301	35 ± 4^{c}
	344	14 ± 1^{b}

Values with different letters are significantly different by the LSD test ($p \leq 0.05$)

When wheat bran oil nanoemulsions were used as inhibitors, higher concentrations of AR than of synthetic additives were necessary to achieve similar reductions of enzyme relative activity (Table 5.2). Around 86% of inhibition was obtained when 344 µg AR/mL were added while 3.4 and 50 µg of sulphite and 4-hexylresorcinol respectively were required for the same inhibition effect. AR inhibitory action could be limited since active compounds were contained in the emulsified wheat bran oil, and they were not as readily

available as in the case of the water soluble synthetic compounds evaluated; yet due to the natural character of AR, utilization of high concentrations should not involve any health problems, as is the case of some of the synthetic additives currently used.

4. CONCLUSIONS

Inhibition capacity of wheat bran oil, formulated as nanoemulsions, to inhibit enzymes involved in the melanosis of different food products has been evaluated. The antioxidant activity of the nanoemulsions was higher the higher the AR and polyphenol content of the oil used for their formulation

Wheat bran oil nanoemulsions were effective to reduce the relative activity of both, mushroom tyrosinase and prawn polyphenoloxidase. The inhibitory capacity of the nanoemulsions evaluated increased with nanoemulsion concentration in the reaction medium, for the two enzymatic systems evaluated. Furthermore, the highest inhibitory capacity was found for the nanoemulsion that showed the highest antioxidant activity.

A linear relationship was found between the AR concentration in the reaction medium and the PPO inhibition, suggesting that AR may be responsible of the PPO inhibition capacity of wheat bran oil nanoemulsions.

Similar reductions of PPO activity of synthetic additives as sulphite and 4-hexylresorcinol could be achieved when wheat bran oil nanoemulsions are used as inhibitors agents, although higher concentration of bioactive compounds (AR) are needed in the reaction medium than are in the case of synthetic compounds. In any case, wheat bran oil nanoemulsion is a natural inhibitor free of the toxicity problems presented by sulphite derivates.

The results obtained in this work showed the potential ability of wheat bran oil, conveniently formulated, for the inhibition of melanosis in food products, such as prawn species.

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Conclusions

Conclusiones

CONCLUSIONS

This work has been focused on the extraction of oil with high content on bioactive compounds from wheat bran. Supercritical fluid technology has been applied for the extraction of the oil, which was then characterized and formulated for its application in food matrices.

From the different experimental studies carried out in this work, the following general conclusions can be established:

- Wheat bran, a by-product of wheat milling, may be a valuable raw material for obtaining oil, rich in bioactive compounds and with a significant antioxidant activity.
- Supercritical fluid extraction with pure CO_2, without cosolvents, can be used for the extraction of wheat bran oil. Temperature is the main parameter that influences the process, increasing the extraction yield, alkylresorcinol (AR) and polyphenol content and antioxidant activity of the oil when temperature is increased. Extraction of wheat bran with supercritical CO_2 in two steps, each step performed under different extraction conditions, yields in the second one, an extract concentrated in AR and polyphenols and low fatty acids content.
- Wheat bran oil is mainly composed of fatty acids. Polyunsaturated fatty acids were the main fatty acids found in the wheat bran oil obtained in this work, with a low amount of saturated ones and a significant content of α-linolenic acid. AR, steryl ferulates and tocopherols are the main bioactive compounds of wheat bran oil together with a low content of other phenolic compounds.
- Fresh wheat bran oil is characterized by having a high antioxidant activity and a low level of oxidation, as indicated by the low levels of hydroperoxides and hexanal. In contrast, the acidity index of the wheat bran oil should be reduced by controlling the storage conditions of the raw material and/or by using an online fractionation process with supercritical CO_2 or by adding a soft refining step.
- Wheat bran oil could be stored at ambient temperature and darkness during more than 150 days maintaining its quality. A quite low increase of oxidation and a slight decrease of antioxidant activity and bioactive compounds in the oil during this storage period indicated the good stability of the oil.

- Wheat bran oil can be incorporated into water systems by formulation of nanoemulsions with a low droplet size. These nanoemulsions can be obtained by using a two-step emulsification process (1. high speed blender and 2. high intensity ultrasonic processor) and a surfactant mixture of Span 80 and Tween 80 and can be stored during at least 60 days at temperatures up to 25 °C.
- Nanoemulsions of wheat bran oil are characterized by having a high antioxidant activity and they are able to reduce the activity of enzymes related to melanosis in plants and animals such as mushroom tyrosinase and polyphenoloxidase extracted from prawn species. This fact shows that wheat bran oil nanoemulsions can be suitable for reducing browning of different food products, which is related to their loss of quality and value.

Future challenges proposed are:

- Scaling-up the supercritical wheat bran oil extraction in order to increase the production volume.
- Reducing the oil acidity by improving the wheat bran storage and/or using an on-line supercritical fluid fractionation or a final refining step.
- Studying the application of wheat bran oil and its nanoemulsions in different food products where antioxidants are required, in order to reduce the use of synthetic additives. As an example, work is being actually done in our laboratory to study the inhibition of melanosis in prawn species by immersion of the whole bodies in diluted nanoemulsions of wheat bran oil.
- Evaluation of wheat bran oil bioactive compounds metabolism and absorption and their potential effects as nutraceuticals or as ingredients of functional products.

CONCLUSIONES

El presente trabajo se ha basado en la extracción de aceite de salvado de trigo, el cual se caracteriza por tener un alto contenido en compuestos bioactivos. La tecnología de fluidos supercríticos ha sido empleada para la extracción del aceite que ha sido después caracterizado y formulado para su aplicación en matrices alimentarias.

De los diferentes estudios experimentales que se han llevado a cabo en este trabajo, pueden destacarse las siguientes conclusiones generales:

- El salvado de trigo, subproducto obtenido en la molienda del trigo, puede ser una valiosa materia prima para la obtención de un aceite rico en compuestos bioactivos y con elevada capacidad antioxidante.
- El aceite de salvado de trigo se puede obtener mediante extracción con dióxido de carbono puro utilizando las condiciones de presión y temperatura adecuadas, sin necesidad de utilizar cosolventes. El principal parámetro que influye en el proceso es la temperatura, incrementándose el rendimiento de la extracción, el contenido en alquilresorcinoles (AR) y polifenoles y la actividad antioxidante del aceite cuando la temperatura aumenta. La extracción de salvado de trigo con CO_2 supercrítico en dos etapas, cada una de ellas realizada a determinadas condiciones de extracción, da lugar a un extracto concentrado en AR y polifenoles y con un bajo contenido en ácidos grasos en la segunda etapa.
- El aceite de salvado de trigo está compuesto principalmente por ácidos grasos, de los cuales la mayoría son ácidos grasos poliinsaturados. Este aceite presenta un reducido contentido de ácidos grasos saturados y una cantidad significativa de ácido α-linolénico. Además, en el aceite de salvado de trigo se encuentran otros compuestos bioactivos como AR, ésteres del ácido ferúlico y tocoferoles y en menor cantidad otros compuestos fenólicos.
- El aceite de salvado de trigo se caracteriza por tener una alta actividad antioxidante y un bajo nivel de oxidación, tal y como muestra el bajo contenido en hidroperóxidos y hexanal que presenta tras su extracción. Por el contrario, el índice de acidez del aceite debe ser reducido, para lo cual deben controlarse las condiciones de almacén de la materia prima y/o utilizar un proceso de extracción con fluidos supercríticos y fraccionamiento en serie o añadir una etapa posterior de refinado poco intenso.

- El aceite de salvado de trigo puede almacenarse a temperatura ambiente y en oscuridad durante más de 150 días manteniendo su calidad. El reducido incremento en la oxidación junto con el ligero descenso en la actividad antioxidante y en el contenido en compuestos bioactivos del aceite durante este almacenamiento son indicativos de su calidad.
- El aceite de salvado de trigo puede ser incorporado en matrices acuosas mediante su formulación en nanoemulsiones con un reducido tamaño de partícula. Estas nanoemulsiones pueden obtenerse mediante un proceso en dos etapas (1. agitador de alta velocidad y 2. procesador de ultrasonidos de alta intensidad) y la utilización de una mezcla de los surfactantes Span 80 y Tween 80 y pueden ser almacenadas durante al menos 60 días a temperaturas de hasta 25 ºC.
- Las nanoemulsiones de aceite de salvado de trigo se caracterizan por tener una alta actividad antioxidante y ser capaces de reducir la actividad de enzimas relacionadas con el pardeamiento que tiene lugar en plantas y animales, como son la tirosinasa de champiñón y la polifenoloxidasa extraída de langostino. Esta capacidad indica que las nanoemulsiones de aceite de salvado de trigo pueden ser adecuadas para reducir el pardeamiento de diferentes productos alimentarios y el cual está relacionado con su pérdida de calidad y valor.

Como retos futuros se proponen:

- El cambio de escala del proceso de extracción de salvado de trigo con CO_2 supercrítico para aumentar el volumen de producción.
- Reducir la acidez del aceite mejorando el almacenado del salvado de trigo y/o utilizando fraccionamiento con CO_2 supercrítico o una etapa de refinado.
- Estudiar la aplicación del aceite de salvado de trigo y sus nanoemulsiones en diferentes alimentos, donde los antioxidantes son necesarios, con el objetivo de reducir el uso de aditivos sintéticos. En la actualidad se están realizando en nuestro laboratorio estudios de inmersión de langostino entero en nanoemulsiones de aceite de salvado de trigo convenientemente diluidas con el fin de evaluar su efecto en el pardeamiento de estas especies.
- Evaluar el metabolismo y absorción de los compuestos bioactivos de salvado de trigo y su potencial como nutraceúticos o como ingredientes de productos funcionales.

Appendix A

Supercritical fluid extraction equipment

ABSTRACT

This appendix shows the characteristics of the equipment used in the supercritical fluid extraction of wheat bran: the laboratory plant used in the first chapter and the pilot plant used in the following ones. Both plants are characterized for their high versatility allowing different configurations according to the different requirements that may be needed in each type of experiment.

In this appendix, the specific configuration and operating mode used in this work for extraction of wheat bran oil with supercritical CO_2 is presented.

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1. SUPERCRITICAL FLUID EXTRACTION EQUIPMENT

The basic supercritical fluid extraction (SFE) scheme consists of an extraction vessel where the raw material is placed, a pump which allows the CO_2 to be charged from the cylinder to the extraction system and the separator in which, due to a pressure reduction, the extracts are released from the gaseous medium and collected. Additionally, other elements such as heat exchangers, valves, temperature and pressure controllers and safety elements are installed.

The SFE plants used for the extraction of wheat bran in this work are a lab scale plant and a pilot plant which diagrams and specifications are described below.

1.1. SFE laboratory plant

1.1.1 Configuration and specifications

The SFE lab plant used for the study of the extraction kinetics (Chapter 1) was designed to operate at pressures up to 50 MPa, temperatures up to 120 °C and at CO_2 flow up to 5 kg/h (Figure A.1).

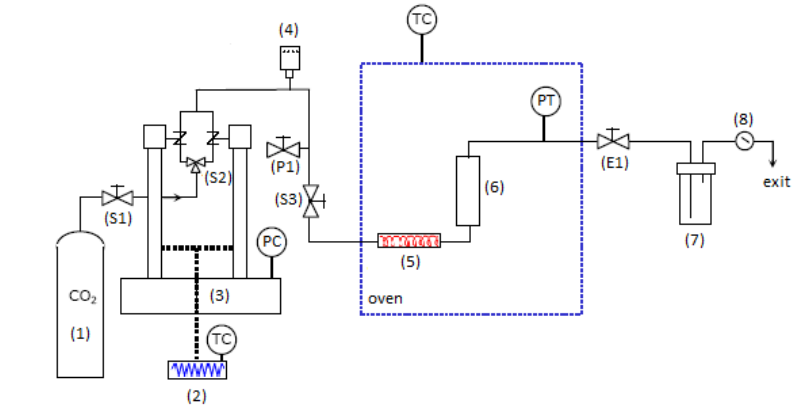

Figure A.1. Flow-sheet of the supercritical fluid extraction laboratory plant

Two syringe pumps (ISCO 260 DM), that work alternatively, provide an uninterrupted flow of liquid CO_2 compressed up to the desired operating pressure. The pressurized CO_2 was pre-heated up to the extraction temperature before entering the extractor which is located in an air oven (Spame, 230V, 50 Hz) where temperature is controlled (± 0.1 K) and measured using a calibrated 4-wires 100 Ω platinum probe. The extractor (40 mL) is equipped with two fritted disks at the ends to allow the CO_2 distribution in all the extractor section and to prevent the solids output. The saturated supercritical fluid that leaves the extractor goes through a needle valve, which allows the decompression of the saturated mixture with the solute precipitation in the separator. The CO_2 leaving the separator, at atmospheric pressure and temperature, is quantified with a thermal mass flow-meter (Mass stream D-5110, M+W instruments).

The lab plant is also equipped with other elements such a cryostat, rupture disks, purge valves, etc.

1.1.2 Operating mode

For the study of the extraction kinetics at different conditions (Chapter 1), 6-8 g of wheat bran (previously sieved or not) were charged in the extractor. Then, CO_2 was pumped until the operational pressure and the oven was set to the extraction temperature before opening the expansion valve and beginning the extraction.

In the case in which static extraction pretreatment was performed, the extractor charged with the raw material and CO_2 at the working pressure and temperature was maintained during a specific time at those conditions before opening the expansion valve and starting the extraction.

For obtaining the extraction kinetics, the separator, where the extract was collected, was weighted at different time intervals until the end of the extraction when the separator was connected to a peristaltic pump (Masterflex model 77390-00, Cole Parmer Instrument Company) during 30 minutes and methanol was recirculated to recover all the extracted compounds that were then analyzed.

1.1.3 References

Murga, R., Sanz, M.T., Beltrán, S., Cabezas, J.L., (2002). Solubility of some phenolic compounds contained in grape seeds, in supercritical carbon dioxide. *The Journal of Supercritical Fluids* 23 (2), 113-121.

1.2 SFE pilot plant

1.2.1 Configuration and specifications

The SFE pilot plant used for the extraction of wheat bran oil in Chapters 2-5 is a home-made plant with high operational versatility. The plant is divided in two operation zones; the high-pressure zone where pressures of 65 MPa and temperatures of 120 °C can be reached and the low-pressure zone which can work up to pressures of 80 MPa and 80 °C (Figure A.2). The CO_2 is pumped from the reservoir using a diaphragm pump with metallic membranes and refrigerated head (Lewa model EH-M-211V1). Solvent flow is regulated by manual adjustment of the stroke length and it could be up to 20 kg/h. A coriolis mass flow-meter (Massflo type Mass 6000, Danfoss) installed before the pump allows monitoring the CO_2 mass flow, volumetric flow, density, and total mass. The extraction vessel is a 2-L autoclave (Table A.1), specially designed and built by Nova Swiss, with an easy open system and a fritted disk that diffuses the supercritical fluid avoiding preferential channels and minimizing the dead volume. Temperature is measured directly inside the vessel, close to the exit, and pressure is measured just after the extractor vessel. The extractor is thermally insulated to keep the processing temperature.

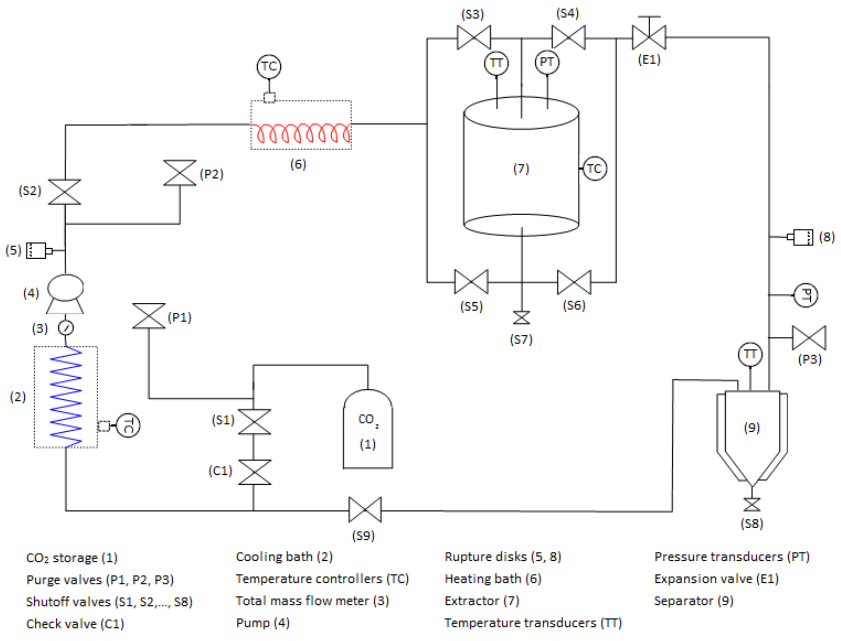

Figure A.2. Flow-sheet diagram of the supercritical fluid extraction pilot plant

Following the extractor, the CO_2 and the extract are separated in a 0.75 L separator vessel built by a local provider (Table A.2) with a conical floor at the bottom to allow the right discharge of the extracts and to reduce the adhesion of the extract to the wall of the vessel. The separator vessel is provided with a heating jacket for temperature control.

Solvent recirculation takes places during all the extraction process until the end of the extraction when the system is depressurized and the raw material and the extract are recovered.

Table A.1. Specifications of the pilot plant pressure vessels

Vessel	Extractor	Separator
Volume (L)	2	0.75
Internal diameter (mm)	100	80
Internal height (mm)	255	150
Wall thickness (mm)	57	22
Maximum pressure (MPa)	70	20
Maximum temperature (°C)	120	80
Closing system	O-ring	Bolted without gasket

A data acquisition system (DAS) is installed to monitor the process. It registers the measurements of the pressure and temperature meters located in different sites of the pilot plant and of the CO_2 flow meter at selected time intervals, allowing an easy representation of the process variables.

Other elements installed in the pilot plant are different types of valves depending on where they are located and their function, heating and cooling systems, and security elements such as rupture disks and a polycarbonate screen which separates the operation and control zones.

1.2.2 Operating mode

The pilot extraction plant was used for the extraction of wheat bran oil in Chapters 2-5. In each run, the extraction vessel was charged with wheat bran (300 g) and then pressurized

with CO_2 at the extraction temperature. In general, extraction time was of 120 minutes and the CO_2 was recirculated after the precipitation of the extract in the separator. At the end of the extraction time, the plant was depressurized releasing the CO_2 to the atmosphere.

For the recovery of the extract, the separator was heated at 50 °C to assure that all the extract was collected when the discharged valve was opened during 15 minutes. This heating was necessary due to the high viscosity of the extract at specific extraction conditions. After the recovery, the co-extracted water was removed either by *vacuum* evaporation at 45 °C during 90 minutes or by centrifugation at 12857g during 30 minutes. Remaining water in the extract was evaluated by drying a sample in an oven at 105 °C until constant weight and it was take into account in the corresponding analytical results.

1.2.3 References

Ganado, O., Beltrán, S., Cabezas, J.L., Cocero, M.J., (2001). Design of a SCFE pilot plant to be operated up to 70 MPa and 475 K and some applications In *2nd International Meeting on High Pressure Chemical Engineering*, Hamburg (Germany).

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Appendix B

Analytical methods

ABSTRACT

This appendix is focused on the analytical methods used for the evaluation of wheat bran oil composition, quality and antioxidant activity. The methods applied for the evaluation of nanoemulsion antioxidant and enzyme inhibitory activities are also presented.

The main objective of this appendix is to extend the analytical methods information, which have been briefly described in Chapters 1 to 4, to facilitate their understanding and practical application. A systematic description of each method and its operational aspects is presented. General lab equipment, reagents or solvents have been omitted while the particular conditions of each analytical method have been emphasized.

INDEX

1. EVALUATION OF OIL COMPOSITION

1.1. Fatty acids content and profile

The fatty acids profile of wheat bran oil was qualitative and quantitative determined by gas chromatography after a methylation step.

Equipment

- GC-FID

Reagents and solutions

- BF_3 12% solution in methanol
- NaOH 0.5 M in methanol
- NaCl saturated solution (36%, w/v)
- Methyl tricosanoate (C23:0, CAS 2433-97-8) 1 mg/mL in isooctane (internal standard)
- Fatty acids methyl esters standards

Sample preparation

The evaluation of fatty acids in wheat bran oil required a previous oil methylation step carried out by reaction with boron trifluoride in methanol and alkali (Figure B.1).

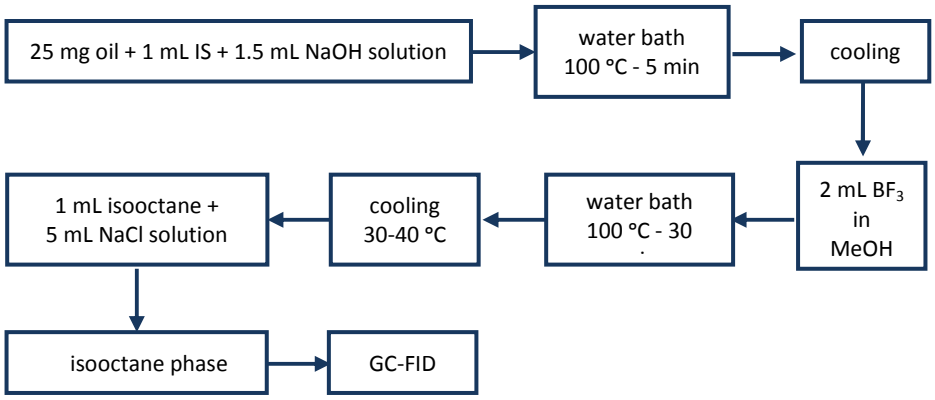

Figure B.1. Methylation process for the analysis of fatty acids in wheat bran oil

Experimental procedure

The fatty acids methyl esters contained in the isooctane phase (section 1.1.3) were analyzed by GC-FID (Table B.1).

Table B.1. Chromatographic conditions for the analysis of fatty acids methyl esters

Column	Omegawax-320, 30 m x 0.32 mm i.d		
Injector temperature	250 °C		
Injection volume	1 µL		
Split ratio	1:50		
Carrier gas and flow	Helium, 1.8 mL/min		
Oven temperature program	°C /min	T (°C)	Hold time (min)
	-	180	20
	1	200	1
	5	220	20
Run time	65 min		
FID temperature	250 °C		

Identification and quantification

Fatty acids were identified by means of standard fatty acids methyl esters (FAME). Quantification was carried out using the corresponding calibration curve of each standard compound, methyl tricosanoate as internal standard and the conversion factor between the methyl ester and the fatty acid in each case (Table B.2). Response factors of those methyl esters that were only available for identification, because they concentration were unknown, were determined by using the linear relationship found between the number of carbons or insaturations of the different fatty acids and their response factors.

Table B.2. Standard compounds and quantification range for fatty acids analysis by GC

Fatty acid (as methyl ester)		CAS	Molecular weight (g/mol)	Quantification range (µM)
Caprylic acid	C8:0	111-11-5	158.24	2.00- 12.0
Capric acid	C10:0	110-42-9	186.29	2.86- 17.3
Lauric acid	C12:0	111-82-0	214.34	4.98- 29.9
Myristic acid	C14:0	124-10-7	242.40	2.20- 13.2
Pentadecanoic acid	C15:0	7132-64-1	256.42	1.23- 7.41
Palmitic acid	C16:0	112-39-0	270.45	8.01- 48.1
Palmitoleic acid	C16:1 n-7	1120-25-8	268.43	3.97- 23.8
Heptadecanoic acid	C17:0	1731-92-6	284.48	1.87- 11.3
Heptadecenoic acid	C17:1 n-7	75190-82-8	282.46	-
Stearic acid	C18:0	112-61-8	298.50	3.63- 21.8
Oleic acid	C18:1 n-9	112-62-9	296.49	12.48- 74.9
Vaccenic acid	C18:1 n-7	6198-58-9	296.49	-
Linoleic acid	C18:2 n-6	112-63-0	294.47	7.36- 44.2
γ-linolenic acid	C18-3 n-6	16326-32-2	292.46	-
α-linolenic acid	C18:3 n-3	301-00-8	292.46	3.65- 21.9
Arachidic acid	C20:0	1120-28-1	326.56	0.97- 5.82
Eicosenoic acid	C20:1 n-9	2390-09-2	324.54	0.98- 5.85
Eicosadienoic acid	C20:2 n-6	2463-02-7	322.53	-
Behenic acid	C22:0	929-77-1	354.61	0.89- 5.36
Erucic acid	C22:1 n-9	112-86-7	338.57	0.94- 5.61
Docosadienoic acid	C22:2 n-6	61012-47-3	350.58	-
Lignoceric acid	C24:0	2442-49-1	382.66	-
Nervonic acid	C24:1 n-9	2733-88-2	380.65	-

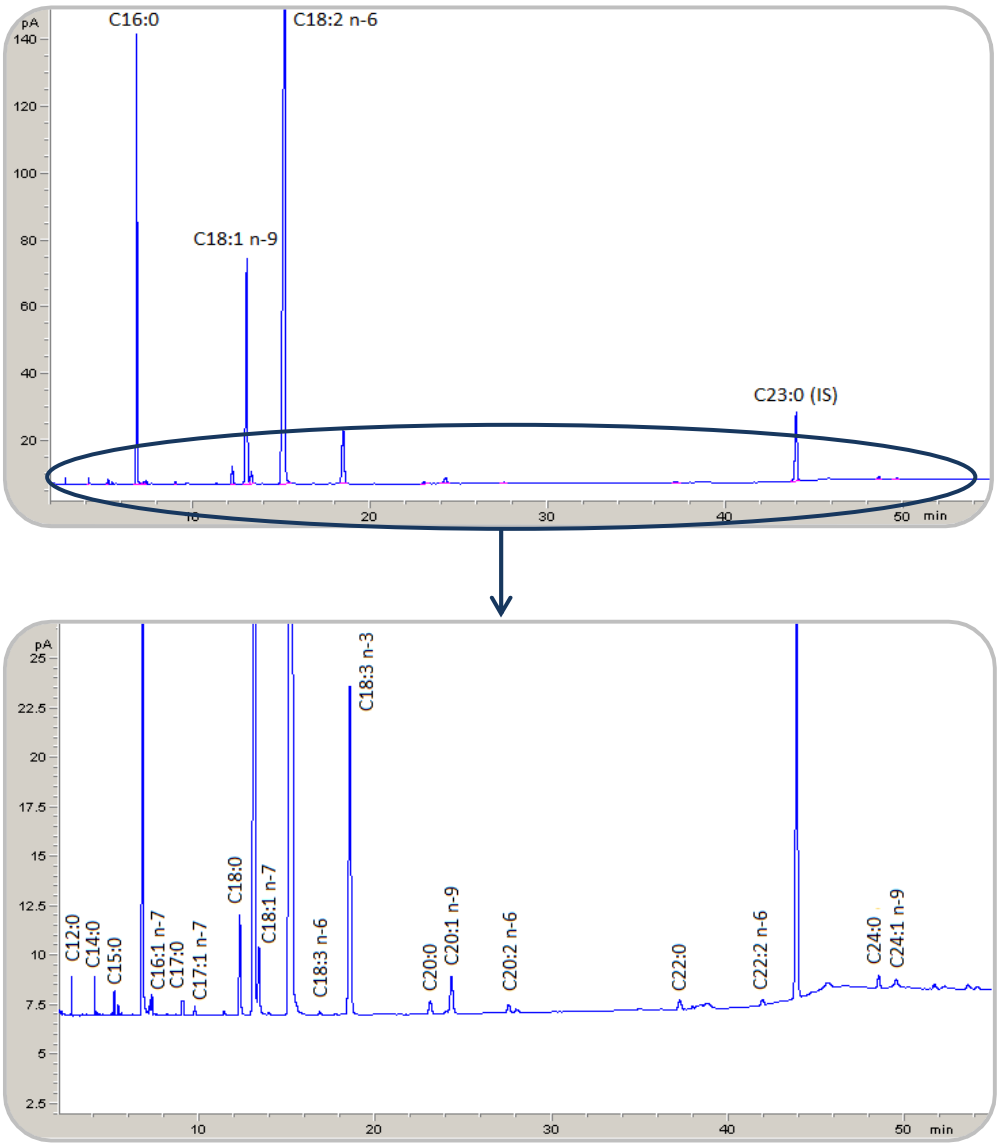

Figure B.2. GC-FID chromatogram of fatty acids in wheat bran (as methyl esters)

References

AOAC, (1995). Fatty acids in encapsulated fish oils and fish oil methyl and ethyl esters. Gas chromatographic method, In: Cuniff, P. (Ed.), *Official Methods of Analysis of AOAC International*. AOAC International, Gaithersburg, MD.

1.2. Alkylresorcinols content and profile

Two methods were used for the evaluation of the AR content in wheat bran oil: a colorimetric method which determines the total AR content and a chromatographic method (HPLC-DAD) which evaluates both, the total content and the individual profile of AR in the sample.

1.2.1 Colorimetric method

The colorimetric method used for the evaluation of the total AR content was based in the measurement of the colored azo-derivates that were produced in the reaction of AR with Fast Blue RR ½$ZnCl_2$ salt in alkaline medium.

Equipment

- Spectrophotometer

Reagents and solutions

- Fast Blue RR salt stock solution 0.05% in methanol
- Fresh solution of Fast Blue RR: 1 part of stock solution + 4 parts of methanol
- K_2CO_3 10% solution in water
- Olivetol (5-pentylresorcinol, CAS 500-66-3)

Sample preparation

Wheat bran oil ethanol solution (5 mg/mL).

Experimental procedure

Wheat bran oil sample was mixed with the Fast Blue fresh solution under alkali conditions and the developed color was spectrofotometrically measured and related with the AR content using olivetol as standard compound (Table B.3).

References

Sampietro, D.A., Vattuone, M.A., Catalán, C.A.N., (2009). A new colorimetric method for determination of alkylresorcinols in ground and whole-cereal grains using the diazonium salt Fast Blue RR. *Food Chemistry* 115 (3), 1170-1174.

Rebolleda, S., Beltrán, S., Sanz, M.T., González-Sanjosé, M.L., (2014). Supercritical fluid extraction of wheat bran oil. Study of extraction yield and oil quality. *European Journal of Lipid Science and Technology* 116 (3), 319-327

Table B.3. Experimental conditions for the quantification of AR by the colorimetric method

Reaction medium	Reaction conditions	Reaction time	Wavelength	Standard and range (mM)
20 µL sample 180 µL methanol 2 mL fresh solution 10 µL K_2CO_3 10%	Ambient	20 min	480 nm	Olivetol 0.28- 2.44

1.2.2 Chromatographic method

Identification and quantification of total and individual AR was performed by using an HPLC-DAD-MS method.

Equipment

- HPLC-DAD-MS

Reagents and solutions

- AR standard compounds

Sample preparation

Wheat bran oil methanol solution (10 mg/mL) filtered with a 0.45 µm syringe filter.

Experimental procedure

AR were analyzed by HPLC-DAD using a C18 column, methanol/water mobile phase and a diode array detector (Table B.4).

Identification and quantification

Identification of AR was carried out according to their mass spectra (Knödler et al., 2008). Positive-ion mass spectra of the column eluate compounds were recorded in the range

m/z 100–500. Nitrogen was used both as the drying gas at a flow rate of 10 L/min and as the nebulizing gas at a pressure of 380 Pa. The nebulizer temperature was set at 350 °C and a potential of 4000 V was used on the capillary.

Quantification of AR was carried out using the calibration curves of the corresponding standard compounds (Table B.5). A linear relationship between the number of carbons of the alkyl chain and the response factor was found (Figure B.3) and it was used to calculate the response factor of the AR that were not available ($C_{27}H_{48}O_2$ (AR-C21) and $C_{29}H_{52}O_2$ (AR-C23)).

Table B.4. Chromatographic conditions for AR analysis by HPLC

Column	Kromasil C18, 250 x 4.6 mm, 5 µm		
Column temperature	25 °C		
Injection volume	100 µL		
Mobile phase	t (min)	A (methanol)	B (water)
	0	98	2
	10	100	0
	50	100	0
Run time	50 min		
Flow rate	0.6 mL/min		
Wash step	Methanol, 25 min, 1 mL/min		
Quantification wavelength	280 nm		

Table B.5. Standard compounds and quantification range for AR analysis by HPLC

Standard compound	Formula	CAS	Molecular weight (g/mol)	Quantification range (µM)
AR-C15	$C_{21}H_{36}O_2$	3158-56-3	320.52	0.62- 312
AR-C17	$C_{23}H_{40}O_2$	41442-57-3	348.57	4.21- 2869
AR-C19	$C_{25}H_{44}O_2$	35176-46-6	376.62	1.24- 1859
AR-C25	$C_{31}H_{56}O_2$	70110-61-1	460.78	14.5- 217

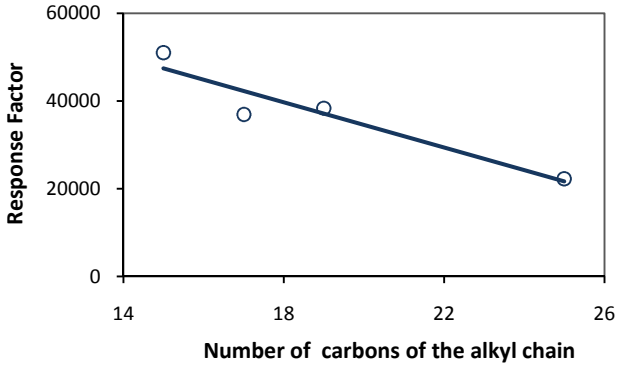

Figure B.3. Relationship between the number of carbons of the alkyl chain and the response factor in HPLC-DAD at 280 nm

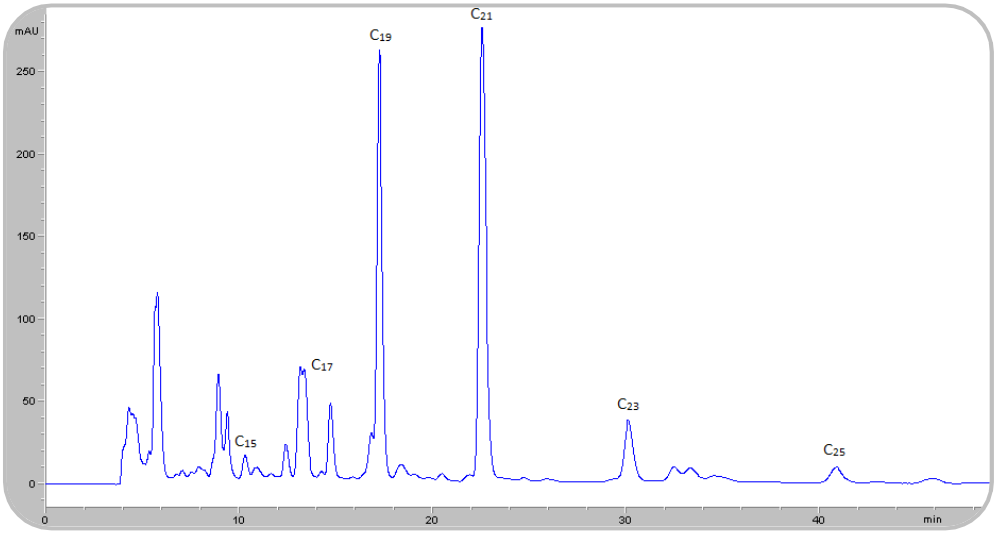

Figure B.4. HPLC-DAD chromatogram of AR in wheat bran oil

References

Knödler, M., Kaiser, A., Carle, R., Schieber, A., (2008). Profiling of alk(en)ylresorcinols in cereals by HPLC-DAD-APcI-MS. *Analytical and Bioanalytical Chemistry* 391 (1), 221-228.

Rebolleda, S., Beltrán, S., Sanz, M.T., González-Sanjosé, M.L., Solaesa, Á.G., (2013). Extraction of alkylresorcinols from wheat bran with supercritical CO_2. *Journal of Food Engineering* 119 (4), 814-821.

1.3. Steryl ferulates content and profile

The ferulic acid esters of sterols (steryl ferulates) of wheat bran oil were determined by using a HPLC-DAD method.

Equipment

- HPLC-DAD

Reagents and solutions

- γ-oryzanol standard mix (CAS 11042-64-1)

Sample preparation

Wheat bran oil methanol solution (10 mg/mL) filtered with a 0.45 µm syringe filter.

Experimental procedure

The oil methanol solutions were used for the quantification of steryl ferulates by HPLC-DAD (Table B.6).

Table B.6. Chromatographic conditions for steryl ferulates analysis by HPLC

Column	Zorbax XDB C18, 150 x 4.6 mm, 5 µm
Column temperature	Ambient
Injection volume	30 µL
Mobile phase	Acetonitrile/methanol/2-propanol (50/40/10)
Run time	30 min
Flow rate	1 mL/min
Quantification wavelength	330 nm

Identification and quantification

Identification and quantification was carried out using a standard mixture of γ-oryzanol and literature data (Hakala et al., 2002). Due to the fact that individual compounds of steryl ferulates were not available, calibration was carried out with γ-oryzanol (33.6-413 mg/L) and each compound was expressed with respect to it.

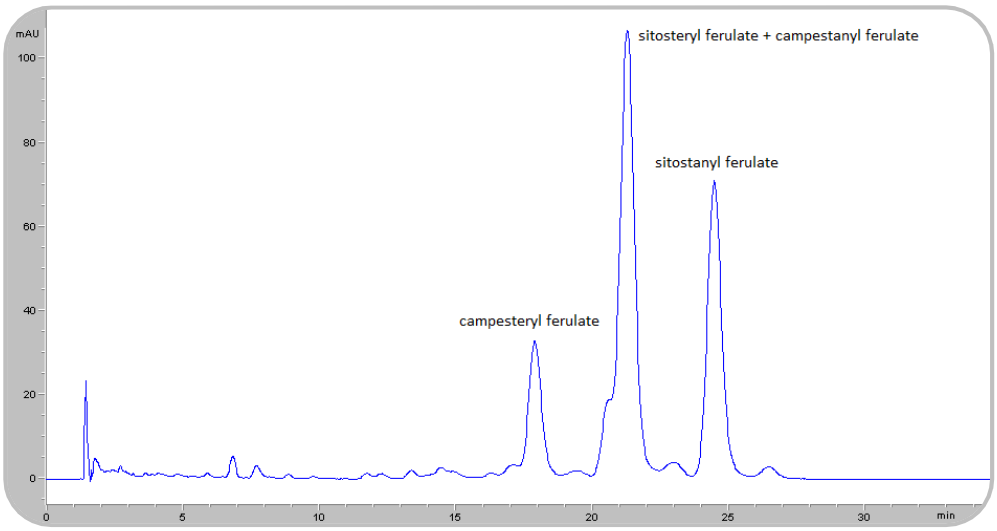

Figure B.5. HPLC-DAD chromatogram of steryl ferulates in wheat bran oil

Hakala, P., Lampi, A.-M., Ollilainen, V., Werner, U., Murkovic, M., Wahala, K., Karkola, S.,Piironen, V., (2002). Steryl phenolic acid esters in cereals and their milling fractions. *Journal of Agricultural and Food Chemistry* 50 (19), 5300-5307.

Mishra, R., Sharma, H.K., Sengar, G., (2012). Quantification of rice bran oil in oil blends. *Grasas y Aceites* 63 (1), 53-60.

1.4. Tocopherol content and profile

The tocopherol content and profile of wheat bran oil was evaluated by HPLC-DAD after their solid phase extraction (SPE).

Equipment

- HPLC-DAD

Reagents and solutions

- *n*-hexane:diethylether (99:1, v/v)
- *n*-hexane:diethylether (98:2, v/v)
- Tocopherol standard compounds

Sample preparation

Tocopherols were extracted from wheat bran oil using SPE silica cartridges (1000 mg/6 mL, Sep- Pak®, Waters) according to Table B.7. The collected fraction was evaporated under reduced pressure at 45 °C and the dry residue obtained was dissolved in 1.5 mL of *n*-hexane.

Table B.7. SPE conditions for extraction of tocopherols from wheat bran oil

Step	Solvent	mL
Conditioning	*n*-hexane	5
Sample loading	Wheat bran *n*-hexane solution 0.1 g/mL	1
Washing	*n*-hexane	5
Elution	*n*-hexane:diethylether (99:1, v/v)	5
	n-hexane:diethylether (98:2, v/v)	50

Experimental procedure

The hexane solution (section 1.4.3) was analyzed by HPLC-DAD for the evaluation of tocopherol content and profile (Table B.8).

Table B.8. Chromatographic conditions for tocopherol analysis by HPLC

Column	ACE 5 silica, 250 x 4.6 mm, 5 µm
Column temperature	Ambient
Injection volume	50 µL
Mobile phase	Hexane/2-propanol (99:1)
Run time	15 min
Flow rate	1 mL/min
Quantification wavelength	296 nm

Identification and quantification

Identification and quantification was carried out using tocopherol standard compounds (Table B.9).

Table B.9. Standard compounds and quantification range for tocopherol analysis by HPLC

Standard compound	Formula	CAS	Molecular weight (g/mol)	Quantification range (µM)
α-tocopherol	$C_{29}H_{50}O_2$	59-02-9	430.71	0.89-597
β-tocopherol	$C_{28}H_{48}O_2$	148-03-8	416.68	1.20-960
γ-tocopherol	$C_{28}H_{48}O_2$	54-28-4	416.68	4.88-976
δ-tocopherol	$C_{27}H_{46}O_2$	119-13-1	402.65	3.70-92.4

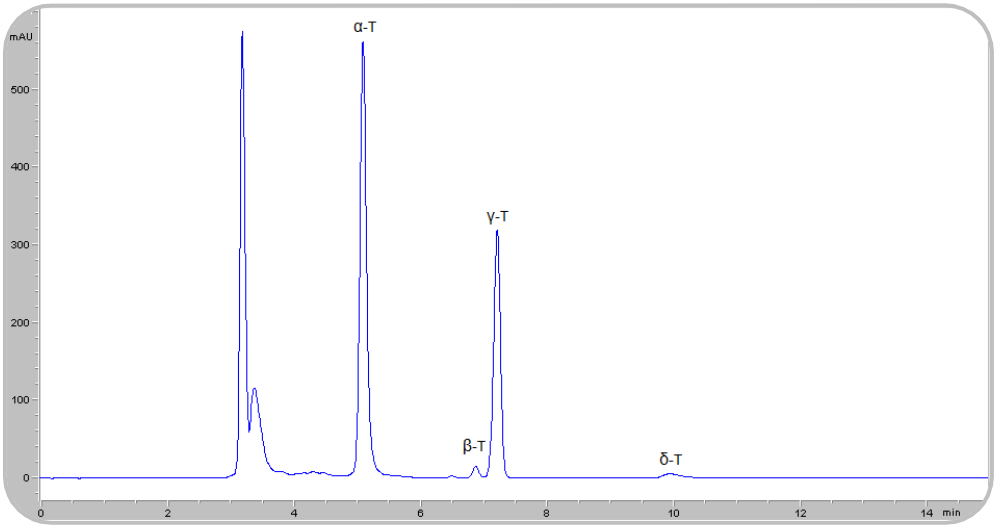

Figure B.6. HPLC-DAD chromatogram of tocopherols in wheat bran oil

References

Grigoriadou, D., Androulaki, A., Psomiadou, E., Tsimidou, M.Z., (2007). Solid phase extraction in the analysis of squalene and tocopherols in olive oil. *Food Chemistry* 105 (2), 675-680.

Rebolleda, S., Rubio, N., Beltrán, S., Sanz, M.T., González-Sanjosé, M.L., (2012). Supercritical fluid extraction of corn germ oil: Study of the influence of process parameters on the extraction yield and oil quality. *The Journal of Supercritical Fluids* 72 (0), 270-277.

1.5. Phenolic compounds content and profile

The phenolic content of wheat bran oil was evaluated by two methods; a simple and fast spectrophotometric index and a chromatographic method with HPLC.

1.5.1 Total polyphenol index

The total polyphenol index (TPI) is based on the maximum absorption that many polyphenols show at 280 nm and can be used as indicative of the polyphenol content of the samples.

Equipment

- Spectrophotometer

Reagents and solutions

- Ethanol

Sample preparation

Ethanol solutions of wheat bran oil (5 mg/mL) were diluted with ethanol (1:10).

Experimental procedure

Absorbance of wheat bran oil diluted samples were measured at 280 nm and results were expressed as absorbance units/g oil.

1.5.2 Chromatographic method

Phenolic compounds present in the wheat bran oil were firstly extracted and then identified and quantified by HPLC- DAD.

Equipment

- HPLC-DAD

Reagents and solutions

- Phenolic standard compounds

Sample preparation

Phenolic compounds were extracted from wheat bran oil according to the following protocol (Figure B.7).

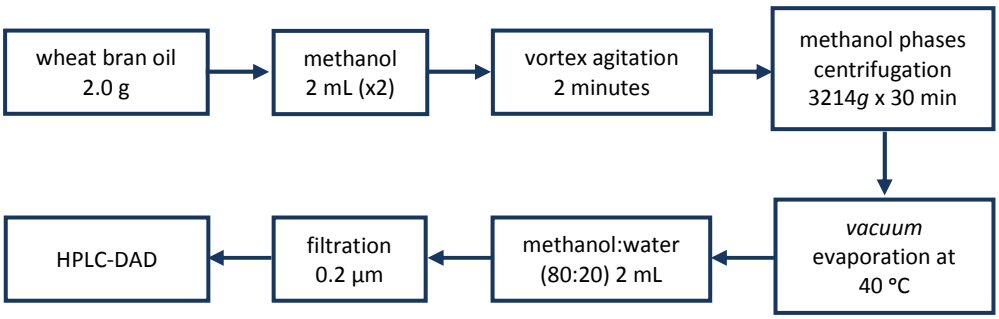

Figure B.7. Protocol for the extraction of phenolic compounds from wheat bran oil

Experimental procedure

Evaluation of phenolic content and profile was carried out by HPLC-DAD (Table B.10).

Identification and quantification

Identification and quantification was carried out by using phenolic standard compounds and UV-VIS spectra data (Table B.11).

Table B.10. Chromatographic conditions for phenolic compounds analysis by HPLC

Column	Spherisorb ODS2, 250 x 4.6 mm, 3 µm		
Column temperature	Ambient		
Injection volume	200 µL		
Mobile phase	t (min)	A (water/acetic acid (98:2))	B (water/acetonitrile/ acetic acid (78:20:2))
	0	100	0
	25	75	25
	60	30	70
	100	0	100
	120	0	100
Run time	120 min		
Flow rate	0.6 mL/min		
Wash step	t (min)	B (water/acetonitrile/ acetic acid (78:20:2))	C (acetonitrile)
	120	100	0
	130	0	100
	140	0	100
Quantification wavelength	200-400 nm		

Table B.11. Standard compounds and quantification wavelength and range for phenolic compounds analysis

Standard compound	Formula	CAS	Molecular weight (g/mol)	Quantification	
				Wavelength (nm)	Range (µM)
Vanillin	$C_8H_8O_3$	121-33-5	152.15	280	28.3-410
Vanillic acid	$C_8H_8O_4$	121-34-6	168.15	254	19.8-248
Syringic aldehyde	$C_9H_{10}O_4$	134-96-3	182.17	320	9.88-190
Ferulic acid	$C_{10}H_{10}O_4$	537-98-4	194.18	320	0.69-96.3
Syringic acid	$C_9H_{10}O_5$	530-57-4	198.17	280	0.22-101
p-OH-benzaldehyde	$C_7H_6O_2$	123-08-0	122.12	280	0.18-287

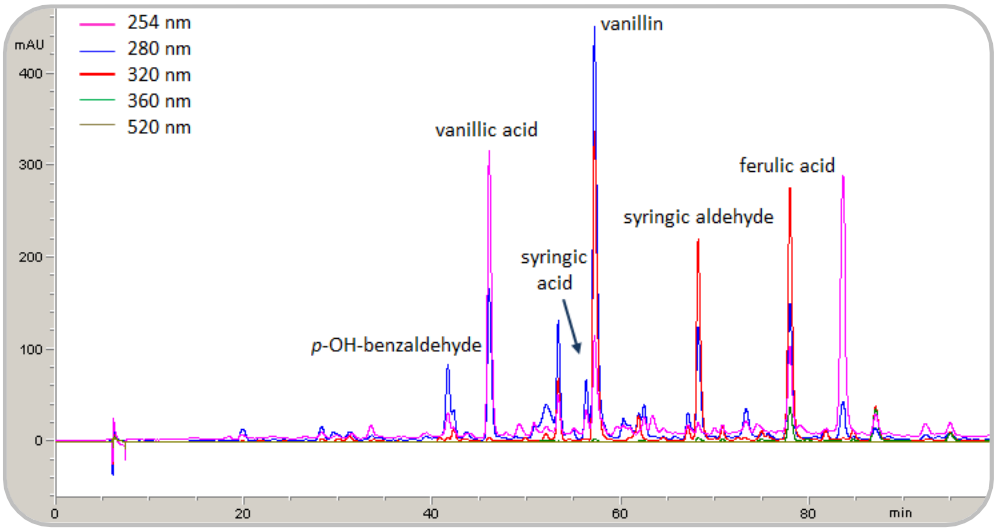

Figure B.8. HPLC-DAD chromatogram of phenolic compounds in wheat bran oil

References

Owen, R.W., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder, B., Bartsch, H., (2000). Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food and Chemical Toxicology* 38 (8), 647-659.

Pérez-Magariño, S., Ortega-Heras, M., Cano-Mozo, E., González-Sanjosé, M.L., (2009). The influence of oak wood chips, micro-oxygenation treatment, and grape variety on colour, and anthocyanin and phenolic composition of red wines. *Journal of Food Composition and Analysis* 22 (3), 204-211.

2. DETERMINATION OF OIL QUALITY PARAMETERS

2.1. Acidity value (AV)

The acidity value is a measure of the amount of carboxylic acid groups in a sample, such as free fatty acids, and it is defined as the amount of potassium hydroxide required for the neutralization of one gram of oil.

Equipment

- Automatic titrator with a pH electrode

Reagents and solutions

- KOH 0.1 N solution in ethanol
- Ethanol 95%:diethyleter (50:50, v/v)
- Potassium hydrogen phthalate (CAS 877-24-7) dried at 105 °C overnight and cooled down during 1 h.

Experimental procedure and calculations

KOH standardization

The KOH 0.1 N solution factor (*f*) was calculated by titration of potassium hydrogen phthalate water solution (4 mg/mL) with the KOH 0.1 N solution using the automatic titrator with pH electrode.

The factor of the KOH solution was calculated according to Equation B.1:

$$f = \frac{m}{V*20.423} \qquad \text{(Eq.B.1)}$$

where m is the potassium hydrogen phthalate mass (mg) and V, the mL of KOH 0.1 N solution.

Evaluation of oil acidity

For the evaluation of wheat bran oil acidity, 1 g of oil was dissolved in 75 mL of the ethanol:diethyleter solution for its titration with the KOH 0.1 N solution using the automatic titrator with pH electrode.

The AV, express as percentage of oleic acid, was calculated according to Equation B.2:

$$AV = \frac{(V_s - V_b) * N * 282.5}{10 * m} \quad \text{(Eq.B.2)}$$

where V_s and V_b are the mL of KOH solution for the sample and blank, respectively, N the normality of the KOH solution and m the weight of oil (g).

References

AOCS, (1990). Official Methods and Recommendation Practices of the American Oil Chemists' Society. American Oil Chemists´ Society, Champaign, IL.

2.2. Peroxide Value (PV)

The peroxide value is used for determining the peroxide oxygen (especially hydroperoxides) in the sample and it is indirectly evaluated by measuring the amount of iodine formed in the reaction of peroxides with iodide ion.

Equipment

- Automatic titrator with a platinum electrode

Reagents and solutions

- Chloroform
- Glacial acetic acid
- Freshly prepared potassium iodide (KI) solution 30% (w/v)
- Sodium thiosulphate ($Na_2S_4O_6$) 0.01 N solution
- Potassium iodate (KIO_3, CAS 7758-05-6) dried at 105 °C overnight and cooled down during 2 h
- H_2SO_4 25% solution

Experimental procedure and calculations

<u>Standardization of sodium tiosulphate 0.01 N solution</u>

Potassium iodide (5 mg) was dissolved with 80 mL of water and mixed with 0.8 mL of KI and 10 mL of H_2SO_4 solution. Then, it was titrated with the sodium thiosulphate 0.01 N solution using the automatic titrator with platinum electrode.

The sodium thiosulphate 0.01 N solution factor (*f*) was calculated according to Equation B.3:

$$f = \frac{m}{V*0.3567} \quad \text{(Eq.B.3)}$$

where m is the potassium iodide mass (mg) and V, the mL of sodium thiosulphate 0.01 N solution.

Evaluation of oil peroxide value

For the evaluation of wheat bran oil peroxide value, 1 g of oil was dissolved in 10 mL of chloroform. Then, 15 mL of glacial acetic acid and 1 mL of KI solution were added. The mixture was vigorously agitated during 1 minute and stored during 5 minutes in darkness.

Finally, 75 mL of distilled water were added and titration with the sodium thiosulphate 0.01 N solution was performed using the automatic titrator with platinum electrode.

The peroxide value, expressed as milliequivalents of O_2 per kg of oil, was calculated according to Equation B.4:

$$PV = \frac{(V_s - V_b)*N}{m} * 1000 \quad \text{(Eq.B.4)}$$

where V_s and V_b are the mL of sodium thiosulphate 0.01 N solution for the sample and blank, respectively, N the normality of the sodium thiosulphate 0.01 N solution and m the weight of oil (g).

References

AOCS, (1990). Official Methods and Recommendation Practices of the American Oil Chemists' Society .American Oil Chemists´ Society, Champaign, IL.

2.3. Hexanal content

The hexanal content of wheat bran oil, which is related with the secondary oxidation of fats, was evaluated by GC after solid phase dynamic extraction (SPDE) of the sample headspace (HS).

Equipment

- HS-SPDE autosampler
- Coated SPDE-syringe with a non-polar 90% polydimethylsiloxane and 10% activated carbon sorbent
- GC-MS

Reagents and solutions

- Hexanal standard (CAS 66-25-1)
- Cyclopentanone (CAS 120-92-3) 0.1% (v/v) solution in ethyl acetate (internal standard)

Sample preparation

Wheat bran oil (0.3 g) was transferred to headspace glass vials and 5 µL of the internal standard were added. A pre-equilibration step of 446 seconds at 55 °C was carried out. The coated needle, connected to a 2.5 mL gastight syringe performed 60 extraction cycles of 2500 µL each, at a speed of 100 µL/s. The SPDE-syringe was then removed from the sample vial and immediately inserted into the "gas station" port where 1000 µL of carrier gas (helium) were pulled into the SPDE-syringe for compounds desorption during 30 seconds and pumped into the GC-inlet at 17 µL/s.

Experimental procedure

The volatile organic compounds previously extracted (section 2.3.3) were injected into the gas chromatograph for the evaluation of hexanal content in the oil (Table B.12).

Identification and quantification

Hexanal identification was achieved by comparison of mass spectral data (Wiley 7th and NIST 05 libraries) and calculated retention indices with those in the literature. Quantification was carried out using the internal standard and a calibration curve of hexanal (3-56 ppb).

Table B.12. Chromatographic conditions for the analysis of hexanal by GC

Column	Carbowax-20M, 60 m × 0.32 mm i.d		
Injector temperature	250 °C		
Injection mode	Splitless		
Carrier gas and flow	Helium, 1.0 mL/min		
Oven temperature program	°C /min	T (°C)	Hold time (min)
	-	35	5
	3	150	-
Run time (min)	43.3 min		
MS conditions	EI mode at 70 eV, voltage multiplier: 1835 V		

References

Corcuera-Tecedor, M.E., (2013). Estudio de la aptitud de un aroma natural obtenido a partir de subproductos de buey de mar (*Cancer pagurus*) para su aplicación en distintas matrices alimentarias, PhD thesis, *Dept. of Biothecnology and Food Science*. University of Burgos, Burgos.

3. EVALUATION OF THE ANTIOXIDANT ACTIVITY IN OILS AND NANOEMULSIONS

3.1. DPPH assay

The DPPH assay is based on the ability of antioxidants to reduce the DPPH radical to the corresponding hydrazine which could be related to the absorbance decrease at 517 nm. The main antioxidant mechanism in this reaction is the single electron transfer (SET).

Equipment

- Spectrophotometer

Reagents and solutions

- DPPH (2,2-diphenil-1-picryl-hydrazyl) 50.7 µM solution
- Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-carboxylic acid, CAS 53188-07-1)

Sample preparation

Wheat bran oil ethanol solution (5 mg/mL) or nanoemulsion without dilution.

Experimental procedure

The sample was mixed with the DPPH solution, and the color reduction was spectrophotometrically evaluated and related to the antioxidant concentration using Trolox as standard compound (Table B.13).

Table B.13. Experimental conditions for the DPPH assay

Reaction medium	Reaction conditions	Reaction time	Wavelength	Standard and range (mM)
20 µL sample 980 µL DPPH solution	Ambient T and darkness	60 min	517 nm	Trolox 0.05-1

References

Brand-Williams, W., Cuvelier, M.E., Berset, C., (1995). Use of a free radical method to evaluate antioxidant activity. *Food Science and Technology* 28 (1), 25-30.

Rebolleda, S., Beltrán, S., Sanz, M.T., González-Sanjosé, M.L., Solaesa, Á.G., (2013). Extraction of alkylresorcinols from wheat bran with supercritical CO_2. *Journal of Food Engineering* 119 (4), 814-821.

3.2. FRAP assay

The ferric reducing antioxidant power (FRAP) assay measures the reduction of the TPTZ reagent by the sample antioxidants what yields a chromophore compound with maximum absorption at 593 nm. This assay is based in the single electron transfer (SET) mechanism.

Equipment

- Spectrophotometer

Reagents and solutions

- Acetate buffer 0.3 M pH 3
- HCl 40 mM solution in milli-Q water
- Freshly prepared TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine) 10 mM solution in HCl 40 mM
- Freshly prepared $FeCl_3$ 20 mM solution in milli-Q water
- TPTZ reagent: 25 mL of acetate buffer 0.3 M + 2.5 mL of TPTZ 10 mM + 2.5 mL $FeCl_3$ 20 mM + 3 mL milli-Q water
- $FeSO_4$

Sample preparation

Wheat bran oil ethanol solution (5 mg/mL) or nanoemulsion without dilution.

Experimental procedure

The reducing power of the sample was evaluated by its reaction with the TPTZ reagent under controlled temperature (Table B.14).

References

Benzie, I.F.F., Strain, J.J., (1996). The Ferric Reducing Ability of Plasma (FRAP) as a measure of ‘‘antioxidant power’’: The FRAP assay. *Analytical Biochemistry* 239 (1), 70-76.

Rebolleda, S., Beltrán, S., Sanz, M.T., González-Sanjosé, M.L., Solaesa, Á.G., (2013). Extraction of alkylresorcinols from wheat bran with supercritical CO_2. *Journal of Food Engineering* 119 (4), 814-821.

Table B.14. Experimental conditions for the FRAP assay

Reaction medium	Reaction conditions	Reaction time	Wavelength	Standard and range (mM)
30 µL sample 970 µL TPTZ reagent	Water bath at 37 °C	30 min	593 nm	Fe (II) ($FeSO_4$) 0.19-1.78

3.3. ABTS assay

The ABTS assay, also called TEAC (Trolox Equivalent Antioxidant Capacity), uses an intensely colored cation radical of ABTS to test the ability of antioxidants to quench radicals. The antioxidant mechanism of this assay is a combination of electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms.

Equipment

- Spectrophotometer

Reagents and solutions

- ABTS 7 mM solution in milli-Q water
- Potassium persulfate ($K_2S_2O_8$) 2.45 mM solution in milli-Q water
- $ABTS^{\bullet+}$ radical solution: this solution is produced by reaction of ABTS 7 mM and potassium persulfate 2.45 mM solutions (1:1, v/v) during at least 16 h in darkness. Then, the radical solution was diluted until the absorbance at 734 nm was around 0.7.
- Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-carboxylic acid, CAS 53188-07-1)

Sample preparation

Wheat bran oil ethanol solution (5 mg/mL) or nanoemulsion diluted with water (1:4).

Experimental procedure

Decoloration of $ABTS^{\bullet+}$ radical solution was spectrophotometrically measured after its reaction with the sample and it was related to the sample antioxidant activity by using Trolox as standard compound (Table B.15).

Table B.15. Experimental conditions for the ABTS assay

Reaction medium	Reaction conditions	Reaction time	Wavelength	Standard and range (mM)
20 µL sample 980 µL $ABTS^{\bullet+}$ solution	Ambient	20 min	734 nm	Trolox 0.1-1.3

References

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Min Yang, A., Rice-Evans, C., (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine* 26 (0), 1231-1337.

Rivero-Pérez, M.D., Muñiz, P., González-Sanjosé, M.L., (2007). Antioxidant profile of red wines evaluated by total antioxidant capacity, scavenger activity, and biomarkers of oxidative stress methodologies. *Journal of Agricultural and Food Chemistry* 55 (14), 5476-5483.

Rebolleda, S., Beltrán, S., Sanz, M.T., González-Sanjosé, M.L., (2014). Supercritical fluid extraction of wheat bran oil. Study of extraction yield and oil quality. *European Journal of Lipid Science and Technology* 116 (3), 319-327.

4. EVALUATION OF THE INHIBITORY CAPACITY OF OIL NANOEMULSIONS ON POLYPHENOLOXIDASE ENZYMES

Anti-melanosis activity of wheat bran oil nanoemulsions was evaluated by using two different enzyme systems, commercial mushroom tyrosinase and prawn polyphenoloxidase extract.

4.1. Tyrosinase inhibitory capacity assay

The *o*-diphenolase activity of commercial tyrosinase was monitored in presence or absence of nanoemulsion to evaluate their capacity to inhibit the enzyme activity.

Equipment

- Microplate reader

Reagents and solutions

- Phosphate buffer 100 mM pH 7
- Mushroom tyrosinase (EC 1.14.18.1) 0.2 mg/mL solution in phosphate buffer
- L-DOPA (3,4-dihydroxy-L-phenylalanine) 1.05 mM solution in phosphate buffer

Sample preparation

Nanoemulsion diluted with water to different concentrations.

Experimental procedure

The nanoemulsions were mixed with the DOPA and enzyme solutions and absorbance at 490 nm was monitored (Table B.16).

Table B.16. Experimental conditions for the tyrosinase inhibition assay

Reaction medium			Wavelength	Monitoring time
Compound	Volume (µL)	Concentration		
Sample	5	25 µL/mL		
DOPA	95	0.5 mM	490 nm	5 minutes
Enzyme	100	1 mg/mL		

Calculations

The initial reaction rate in the presence or absence of sample was calculated from the slope of the reaction curve (absorbance vs time) and the relative activity and inhibition were calculated (Eq.B.5 and B.6).

$$Relative\ activity\ (\%) = \frac{Vi}{Vo} * 100 \quad \text{(Eq.B.5)}$$

$$Inhibition\ (\%) = 100 - relative\ activity \quad \text{(Eq.B.6)}$$

where Vi and Vo are the initial reaction rates in the presence or absence of sample respectively.

References

Chen, Q.-X., Song, K.-K., Qiu, L., Liu, X.-D., Huang, H., Guo, H.-Y., (2005). Inhibitory effects on mushroom tyrosinase by *p*-alkoxybenzoic acids. *Food Chemistry* 91 (2), 269-274.

4.2. Prawn polyphenoloxidase inhibitory capacity assay

For the evaluation of the inhibitory effect of wheat bran oil nanoemulsions on the polyphenoloxidase (PPO) activity, an enzymatic extract from prawn cephalothoraxes was obtained. The enzymatic activity of this extract in the presence or absence of nanoemulsion was evaluated.

4.2.1 Preparation of PPO extract from the cephalothoraxes of prawn

Equipment

- Grinder
- Refrigerated centrifuge
- Dialysis tubes (Novagen D-Tube dyalizers, 12-14 kDa, Millipore Billerica, MA)

Reagents and solutions

- Liquid nitrogen
- Sodium phosphate buffer pH 7.2
- Extracting buffer: 0.05 M sodium phosphate buffer pH 7.2, containing 1.0 M NaCl and 0.2% Brij-35
- Ammonium sulphate ($(NH_4)_2SO_4$)

Experimental procedure

The prawn cephalothoraxes were separated, pooled and powdered by grinding with liquid. The power obtained (50 g) was used for the extraction of PPO (Figure B.9).

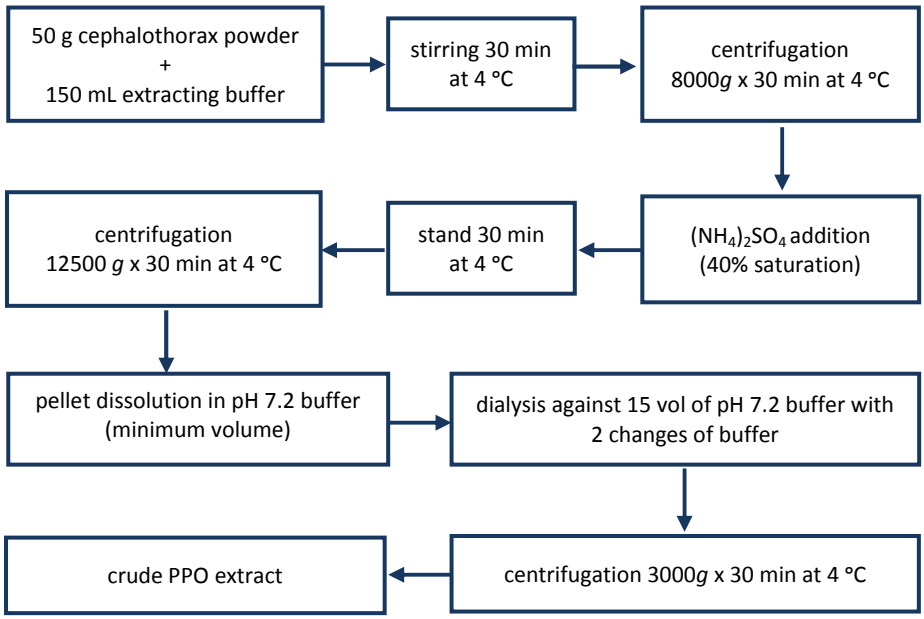

Figure B.9. Protocol for the extraction of PPO from prawn cephalothoraxes

4.2.2 Measurement of PPO inhibition capacity

Equipment

- Spectrophotometer

Reagents and solutions

- Phosphate buffer pH 6
- PPO extract (section 4.2.1)
- L-DOPA (3,4-dihydroxy-L-phenylalanine) 15 mM solution incubated at 45 °C

Sample preparation

Nanoemulsions pH was adjusted to 6 by using 0.1 N NaOH and they were diluted with the assay buffer at 45 °C to obtain the different concentrations just before the assay.

Experimental procedure

The PPO extract was mixed with the sample and the DOPA solution (45 °C) and the absorbance at 475 nm was monitored (Table B.17).

Table B.17. Experimental conditions for the PPO inhibition assay

Reaction medium			Wavelength	Monitoring time
Compound	Volume (mL)	Concentration		
PPO extract	0.2	83 µL/mL		
Sample	1.0	416 µL/mL	475 nm	3 minutes
DOPA	1.2	7.5 mM		

Calculations

The initial reaction rate in the presence or absence of sample was calculated from the slope of the reaction curve (absorbance vs time) and the relative activity and inhibition were calculated (Eq. B.5 and B.6).

$$Relative\ activity\ (\%) = \frac{Vi}{Vo} * 100 \qquad \text{(Eq. B.5)}$$

$$Inhibition\ (\%) = 100 - relative\ activity \qquad \text{(Eq. B.6)}$$

where Vi and Vo are the initial reaction rates in the presence and absence of sample respectively.

References

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