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SUPERCRITICAL CO_2 EXTRACTION OF WHEAT BRAN OILS

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1 Introduction

1.1 Solvent extractions

Separation of components and active ingredients is a very common procedure in nowadays food, cosmetics and pharmaceutical industries. The most common way of extraction is by using organic solvents such as toluene, propane, hexane, petroleum ether, chloroform, acetone, etc. These kinds of extractions are relatively cheap to carry out, but often require of relatively high temperatures. These can end up degrading the most thermolabile compounds such as proteins or antioxidants and additionally, can leave rests of solvents in the final product. An alternative to these methods is to use Supercritical Fluid Extraction (SFE). Its application has grown steadily due to several advantages over classic organic solvent extractions [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

1.1.1 Supercritical $C0_2$ extraction

A component is thought to be in a supercritical state when its temperature and pressure are higher than the critical values. At such critical conditions there are no sudden changes in the component properties. Once crossing the critical point, the fluid properties do not change significantly with the process conditions yet the magnitude of variation of the effects in the solutes and reactants can be tremendous [BRUNNER, 1994].

CO_2 is the most widely used supercritical fluid because of its properties. It is non-flammable, non-corrosive and non-toxic, inert to most materials and additionally cheap and easy to obtain in larger amounts with high purity. Moreover, it has a relatively low critical temperature (31.1 ºC) making it suitable for the extraction of heat sensitive bioactive compounds and reducing the energy costs within the process. CO_2 extraction allows modifying the temperature and pressure within the process, which can improve its efficiency or increase the selectivity and yield of specific compounds [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

Numerous studies have been done using supercritical CO_2 to do all sorts of extractions (lipids, waxes, bioactive compounds, etc.) out of very diverse raw materials (vegetables, fruits, cereals and their by-products, wood, animal products, etc.) or as a pre-treatment to make further transformations of such raw materials more efficient [ROOZBEH et al., 2010].

With this type of technology it is also possible to obtain different fractions of the oil by applying different temperatures and pressures as the different fatty acids have different melting points and solubilities. Several studies were carried out in the past years, in which C02 was an effective alternative technique to produce high value oil out of different raw

materials [BALACHADRAN et al., 2008; CHAO-RUI et al., 2008; CHICH-HUNG et al., 2008; DANIELSKI et al., 2005; GO-WOON et al., 2010; KYUNG-TAE et al., 2010; PERRETTI et al., 2003; PIRAS et al. 2009; SARMENTO et al., 2006; SHAO TONG and LI YA, 2011; SPARKS et al., 2006; KUK and DOWD, 1998; RAMSAY et al., 1991].

1.1.1.1 The extraction equipment

The equipment necessary to do extractions depends very much on the nature of the process and the extraction, the raw materials and solvent used and mostly if the process is going to be carried out with the solvent being cycled under supercritical (gasous) or near-critical (liquid) conditions. The equipment used consists basically of a pump or a compressor, an extractor, a heat exchanger and a separator. Sometimes, the solubility of low volatile substances in supercritical solvents may be too low for carrying out the extraction and there is the need to use a co-solvent such as ethanol, methanol, propane or petroleum ether, which will increase the density and can further enhance concentration in the supercritical solvent by molecular interactions. Co-solvents can be in liquid or gas form (for example: ethanol and propane respectively). In those cases using a co-solvent, the composition of the mixture has to be adjusted previous to the access to the extractor [BRUNNER, 1994].

The energy consumption in the process will be related to the pressure. At pressures higher than 30 MPa, those equipments using a compressor will require more energy whilst under this pressure value it will be those using a pump the ones using more energy. Pump processes have a better control of the solvent mass flow but they require of several heat exchangers and a condensator. In both the case of carrying a supercritical or near-critical process both a pump or a compressor can be used but it will be important to choose that one which will result in higher economic and energy savings. Pumps require usually of a lower economic investment than compressors, and considering that supercritical processes are carried out at higher pressures, in such conditions a pump may be more interesting and viable.

Once the solute has been dissolved by the solvent at the extractor, it is then necessary to separate the desired dissolved substances from the supercritical solvating fluid to obtain the extract and to clean the solvating fluid to be able to reuse it in extraction processes. This is going to be performed at the separator. There are many different methods to do so, but those which add no additional substances to the mix with small changes in the state conditions are usually more interesting for industry. One possible way is to reduce the solvent power. The solvating power of a supercritical fluid will depend on the temperature and pressure conditions, meaning changing those parameters can be used to separate the dissolved substances. Both decreasing the pressure and increasing the temperature lead to a decrease of the fluid density which also determines its lower solvating power. For those cases where the extract is only soluble in the gas phase an effective method is to extend the

extraction period to a point where the conditions are in near critical conditions. In this point there are going to coexist three phases, one gaseous, one liquid and the extract itself, insoluble in the liquid phase. The extract can then easily removed and the rest of the solvent evaporated to be used again. In other occasions it might be advantageous to use a mass separating agent, such as an absorbent, an adsorbent. When using an absorbent, this should dissolve the solute but should not be soluble in the supercritical solvent nor affect the quality of the product. Adsorption is very effective at removing the extract from the solvent gas to be used again in other extractions, but the extracts themselves are difficult to remove from the adsorbent. The interesting property about absorbents and adsorbents is that they allow the process to remain at constant pressure. Other methods of mass separation are the use of a membrane, which acts thanks to the significantly different molecular weight of supercritical solvent and extract, or a substance of low solvent power, which reduces the solubility of the extract in the solvent [BRUNNER, 1994]. A schematic representation of a supercritical CO_2 extracting unit can be seen on Figure 1.

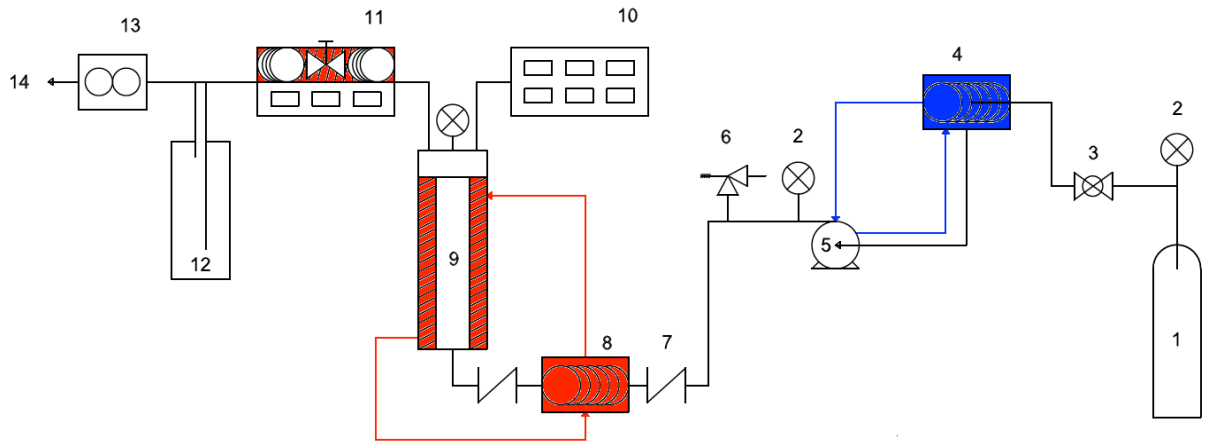


Figure 1: Schematic diagram of a typical construction of a supercritical CO_2 extracting unit.

Legend:

1: CO_2 tank	6: Safety valve	11: Heat exchanger
2: Pressure gauge	7: Check valve	12: Separator
3: On-off valve	8: Water bath	13: Dry gas meter
4: Chiller	9: Extractor	14: Vent
5: Pump	10: Digital thermometer	

1.1.1.2 Supercritical CO_2 extraction vs. conventional methods

Using organic solvents such as toluene, propane, hexane, petroleum ether, chloroform, acetone, etc. are cheap and widely used methods to do extractions. The relatively high temperatures achieved in some of these processes can end up degrading the more thermolabile compounds. Additionally, the use of organic solvents is not very friendly to the environment and leave rests of solvents in the final product, making it susceptible to be harmful to human health. These facts make this technology limited due to the regulations concerning the concentration of toxic residues deriving of organic solvents [GO-WOON et al., 2010; KYUNG-TAE et al., 2010]. Expensive and time-consuming processes such as distillation have to be used to remove such residues. Organic solvents also present safety issues, as the vapors of certain solvents like hexane are flammable and could lead to explosions. Therefore they need to be monitored during oil extraction operations [SPARKS et al., 2006].

Supercritical CO_2 extraction is seen nowadays as a good alternative to fight these problems. CO_2 is non-flammable, non-corrosive and non-toxic, inert to most materials, is cheap and easy to obtain in larger amounts with high purity and its low critical temperature (31.1 ºC) allows the processes to be taken at temperatures near to those in the environment, which reduces the losses of those compounds which are thermolabile such as proteins, antioxidants and other nutritionally valuable components [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

1.1.1.3 Supercritical CO_2 extraction in cereals

Cereals are world widely cultivated grasses of the monocotyledonous family *Gramineae* and have been historically the basis of human nutrition due to their successful production and easy storage. The most important according to the worldwide production are maize, rice, wheat, barley, oats, and rye. Their grains are edible, nutritious and rich in starch, proteins, fat and some vitamins [DENDY and DOBRASZCZYK, 2001]. A huge variety of foodstuffs are made out of the grains, such as pasta or breads, being the average annual consumption of a human person 171 kg [KENT and EVERS, 1994]. In addition, many alcoholic and non-alcoholic drinks are of cereal origin, such as kwas (popular drink in eastern Europe) in the first case or beer and whiskey in the second.

When cereals are milled to obtain the grain that will be used in the production of the above-mentioned products, the straw and the outer layers of the grain (hull, the germ and bran) are removed. This means every year millions of metric tons of cereal origin by-products. One big part of these enormous amounts of by-products are often disposed in landfills because they become rancid. Another fraction is often sold for animal feeding [SPARKS et al., 2006; SARMENTO and FERREIRA, 2006] or as a source of energy using it for the production of ethanol and bio-fuels or by simply burning them [SARMENTO and FERREIRA, 2006; ROOZBEH et al., 2010].

Several studies reflected the interest of using supercritical CO_2 extraction with a cereal by-product such as cereal germ to obtain high value oil as it has the highest tocopherol content of all plant origin oils (around 2500 mg/kg), where α-tocopherol represents up to 60%. It is also rich in unsaturated fatty acids, mostly linoleic and linolenic. Comparing the oil extraction with supercritical CO_2 extraction and organic solvent extraction did not show significant differences in fatty acid composition of the final product depending on the extraction method that had been used [SHAO TONG and LI YA, 2011; PIRAS et al. 2009]. However, SHAO TONG and LI YA (2011), stated in their study that the oil obtained with supercritical CO_2 had a higher radical scavenging ability (up to 96.08% using DPPH) than that one obtained with the organic solvent (petroleum ether in this case), meaning that it had a higher content in bioactive compounds such as tocopherols and other phenolic compounds. Moreover, defatted germ flour with very high content in protein is obtained as a by-product from the oil extraction. In the research of SHAO TONG and LI YA (2011), the levels of protein in the defatted wheat germ were up to 34.3%, making it a very interesting source of vegetable protein. Moreover, the amounts of lysine (2.47%) were remarkable as it is the first limiting amino acid in the grain according to GE et al. (2000). This by-product could, therefore, have a high interest in food fortification or as a high-value feed for farm animals.

Cereal straw has also been subject of study in the field of supercritical fluid extraction with CO_2 [ATHUKORALA, and MAZZA, 2010; ATHUKORALA, and MAZZA, 2011], or using the supercritical CO_2 to do a pre-treatment on such raw material to obtain reducing sugars further on, which are necessary in the ethanol conversion process [ROOZBEH et al., 2010]. ATHUKORALA, and MAZZA (2010; 2011) stated that it is possible to obtain waxes from triticale and flax straws with similar thermal and spectral features to those of the commercial waxes used nowadays in industry, which showed a pathway to obtain alternative materials to synthetic and other natural waxes. Additionally, the process left a by-product consisting of de-waxed straw rich in cellulose which could be used in numerous energy processes (ethanol obtaining) or as a feedstuff.

1.1.1.3.1 Supercritical CO_2 extraction from cereal bran

Cereal bran is part of the outer layers covering cereal grains and includes the pericarp (fruit coat), testa (seed coat), nucellus (hyaline layer) and aleurone layer [KENT and EVERS, 1994; KHAN and SHEWRY, 2009]. It is mostly conformed fiber, fat, protein and water, being the second and the third the most interesting nutritionally speaking and, therefore, for the food industry.

Cereal brans are also rich in natural antioxidants such as carotenoids, phytosterols, tocopherols, tocotrienols, alkylresorcinols, oryzanols, etc. [NYSTROM et al., 2005; ZHOU et al., 2005; HALLIWELL, 1992; CHICH-HUNG et al., 2008; KAMAL-ELDIN et al., 2009; ; KORYCINSKA et al., 2009]. Antioxidants are biologically important in the way that they

prevent important molecules such as DNA, proteins and membrane lipids from the damage of oxidation thus they are reducing the risk of certain diseases such as cancer or cardiovascular diseases [YU et al., 2002].

As many studies have stated so far, it is possible to extract oils from cereal bran which have an important added value thanks to their high content in the natural antioxidants mentioned above [SARMENTO and FERREIRA, 2006; CHICH-HUNG et al., 2008; GO-WOON et al., 2010; KYUNG-TAE et al., 2010; DA CRUZ FRANCISCO et al., 2005].

Several studies made in the past compared the extraction yield of rice bran between using supercritical CO_2 and organic solvents like hexane or propane. The results were very similar, as the oil yield with supercritical CO_2 (at 62 MPa and 100 ºC) was 20.4% and with hexane (at 0.101 MPa and 69 ºC) was 20.5% [KUK and DOWD, 1998]. Another study compared the oil extraction yields of hexane and supercritical CO_2 together with ethanol as a co-solvent being those 20.21% and 17.98 respectively, under conditions of 30 MPa and 35 ºC [RAMSAY et al., 1991]. A third study compared the extraction yields of supercritical CO_2 (at 20 MPa and 85 ºC) and propane (at 0.62 MPa) being those 23.6% and 23.0% respectively for oil yield, and 15.6% and 15.0% respectively for protein yield [SPARKS et al., 2006].

SARMENTO et al. (2006) analyzed the yields of certain antioxidants in supercritical CO_2 oil extractions from rice bran, and stated that under any conditions of extraction, the tocotrienol yields were larger than those of tocols. They also figured out that the higher extraction yields for both tocotrienols and tocols were unde conditions of 200 bar and 40 ºC. CHIH-HUNG et al. (2008) studied the yields of oryzanols in the same product comparing the yields in both supercritical CO_2 and normal extraction with hexane and indicated that they were indeed very similar.

1.1.1.3.1.1 Supercritical CO_2 extraction of wheat bran oil

Wheat is one of the most important agricultural products in the world and it is cultivated almost all over the world as it is adaptable to a wide range of environmental conditions, and is a very important part of the diet in developed countries [KENT and EVERS, 1994]. The larger areas of production are in the northern hemisphere, and the top seven world regions in production volumes in millions of tones are China (93.9), the European Union (91.7), India (68.8), the United States (53.3), the Russian Federation (46.9), Canada (20.6), and Australia (19.4) (ten-year averages, 1993-2002) [KHAN and SHEWRY, 2009]. During its processing, large amounts of bran and other by-products are produced. Wheat bran consists of 14.5 wt% of rough wheat grain [XIE et al., 2008].

Wheat bran is part of the outer layers covering the starchy endosperm and includes all the layers of the pericarp (fruit coat) and some of the seed itself. The pericarp is formed by the outer pericarp and the inner pericarp. The outer pericarp is at the same time conformed

by the layers of the outer epidermis, the hypodermis and the thin-walled cells. The inner pericarp is conformed by the layers of the intermediate cells, the cross cells and the inner epidermis (tube cells). The layers belonging to the seed which are ripped off in the milling process and are part of the bran are the seed coat (testa), the nucellar epidermis (hyaline layer) and the aleurone layer, leaving only the starchy endosperm and the embryo for further processes (see Figure 2) [KENT and EVERS, 1994; KHAN and SHEWRY, 2009].

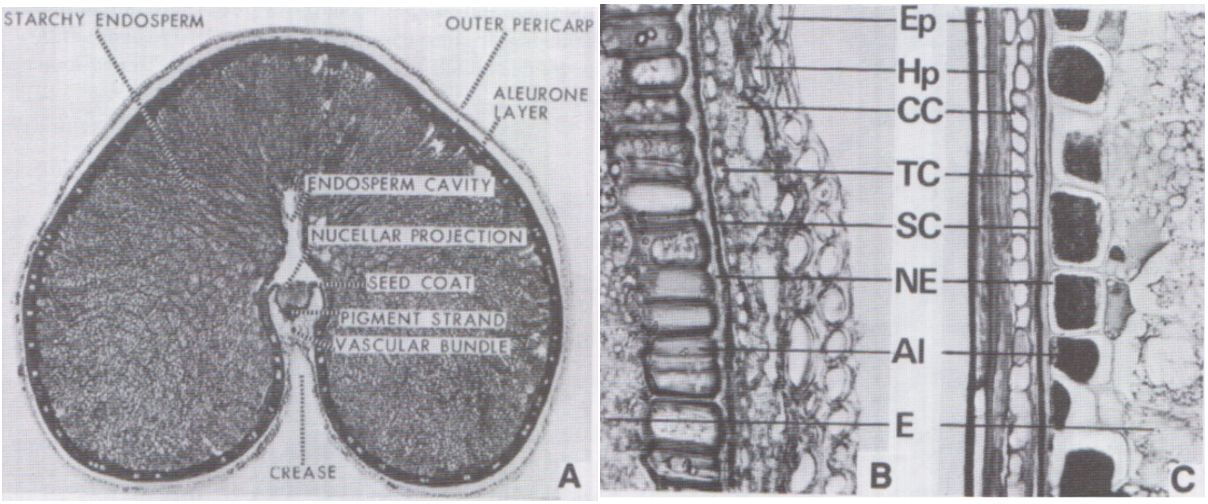


Figure 2: A, transversal photograph of the wheat grain. B (transversal) and C (longitudinal), photomicrographs of outer layers of the wheat grain. Ep=outer epidermis, Hp=hypodermis, CC=cross cell, TC=inner epidermis, SC=seed coat, NE=nucellar epidermis, Al=aleurone layer, E=starchy endosperm [KHAN and SHEWRY, 2009].

A chemical characterization made KAMAL-ELDIN et al. (2009) to two different wheat brans exposed that they had a content in protein of 15.2 and 16.9%, in fats of 5.6%, in starch od 8.8 and 24.8%, in total dietary fibre of 53.1 and 39.9%, in cellulose of 12.1 and 9.3% and in ashes of 6.5 and 5.5%. The rest of their composition consisted of minerals and trace elements, some of them with a significant relevance in human nutrition (see Table 1).

Table 1: On the left, content of proximate components, phytic acid and minerals of two wheat bran samples (g/100 g DM).On the right, contents of vitamins and bioactive components of two wheat bran samples (mg/100 g DM) and relative percentages of individual sterols and alkylresorcinols [KAMAL-ELDIN et al., 2009].

	Wheat 1	*Wheat 2*		*Wheat 1*	*Wheat 2*
Protein [g/100 g DM]	15.2	16.9	**Total tocols**	10.6	8.4
Fat [g/100 g DM]	5.6	5.6	**α-Tocopherol**	0.3	1.5
Starch [g/100 g DM]	8.8	24.8	**β-Tocopherol**	0.1	0.9
Total dietary fibre [g/100 g DM]	53.1	39.9	**α-Tocotrienol**	2.2	1.1
Arabinoxylan [g/100 g DM]	29.8	22.4	**β-Tocotrienol**	8.1	4.9
β-glucan [g/100 g DM]	2.6	2.2	**Total folate**	0.08	0.08
Cellulose [g/100 g DM]	12.1	9.3	**Glycine betaine**	431	441
Klason lignin [g/100 g DM]	4.9	3.3	**Trigonelline**	4.9	3.7
Fructan [g/100 g DM]	3.7	2.8	**Total sterols/stanols**	190	160
Phytic acid [g/100 g DM]	4.19	3.48	**β-Sitosterol**	71.6	66.2
Ash [g/100 g DM]	6.5	5.5	**Campesterol**	23.2	25.8
Phosphorus [mg/100 g DM]	1390	1240	**Stigmasterol**	7.4	6.2
Phytic acid phosphorus [mg/100 g DM]	1180	980	**Sitostanol**	37.4	23.7
Potassium [mg/100 g DM]	1760	1530	**Campestanol**	33.3	25.9
Magnesium [mg/100 g DM]	430	360	**Other sterols**	17.1	12.2
Calcium [mg/100 g DM]	100	100	**Total alkylresorcinols**	400	240
Sodium [mg/100 g DM]	15	8	**C17:0**	16.0	7.2
Manganese [mg/100 g DM]	7	7	**C19:0**	128.0	62.4
Zinc [mg/100 g DM]	6	7	**C21:0**	200.0	136.8
Iron [mg/100 g DM]	9	11	**C23:0**	44.0	26.4
Copper [mg/100 g DM]	1.5	1.4	**C25:0**	12.0	7.2
			Ratio C17:0 / C21:0	0.07	0.05
			Ferulic acid	396	280
			para-Coumaric acid	9.2	7.2
			Total lignans	3.6	2.8

Wheat bran is a good source of dietary fibres, protein, fat and is rich in numerous natural antioxidants such as carotenoids, phytosterols, tocopherols, tocotrienols, alkylresorcinols, etc. [NYSTROM et al., 2005; ZHOU et al., 2005; HALLIWELL, 1992; KAMAL-ELDIN et al., 2009; KORYCINSKA et al., 2009]. Antioxidants have their importance in human metabolism by preventing certain diseases of concern such as cancer or cardiovascular diseases as they scavenge to the free radicals of important molecules such as DNA, proteins and membrane lipids, preventing them from the damage of oxidation [YU et al., 2002].

Some studies made in the past stated that it is possible to extract oils with high content in antioxidants from wheat bran using supercritical (<31.1 ºC) and near-critical (<31.1 ºC) CO_2 extractions, giving the final product an added value. The amount of these antioxidant in the oil extracted is dependent on the extraction conditions. α- and β-tocopherol contents (measured with HPLC) and the total phenolic content (TPC) in general, increase both with increasing temperature and pressure according to these researches. Increasing the pressure increases de density which allows the solvating CO_2 to dissolve more solutes. Increasing the temperature increases the diffusivity and decreases the viscosity which makes the solute more soluble. The total carotenoid content increased as well with increasing pressure for the same reason stated above. At supercritical conditions, the yield increased as well with the increasing temperature even though raising the temperature decreases the density. This could be due to the fact that increasing the temperature increased the solute vapour pressure. However, under near-critical conditions (25 and 30 ºC), the carotenoid yield decreased with the increasing temperature, giving the idea that at these temperatures, the increase of the vapour pressure was not enough to overcome the decrease in the fluid density [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

According to some studies made in the past, wheat bran oil has a weak odour and a slightly yellow colour. The oil yield is dependent on the extraction conditions (temperature, pressure, extraction time, and probably as well, the size of the particles and the water content in the raw material). Increasing the pressure had a positive effect on the oil yield both at near-critical and supercritical conditions, because doing so increases the density of the solvating fluid and thus increasing its solvating power. At supercritical conditions, the oil yield increased as well with the increasing temperature even though raising the temperature decreases the density. This could be due to the fact that increasing the temperature increased the solute vapour pressure. The oil yield decreased with the increasing temperature under near-critical conditions (25 and 30 ºC), which could mean that at near-critical temperatures, the increase of the vapour pressure was not enough to overcome the decrease in the fluid density [GO-WOON et al., 2010; KYUNG-TAE et al., 2010]. [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

According to previous studies, the major fatty acids present in wheat bran oil are in this order linoleic, palmitic, oleic and γ-linolenic. The oils contained highest percentage of linoleic acid independently of the conditions of heat and pressure in the process, in a range between 45.4 and 60%. Palmitic acid was always the second most abundant (and the most abundant within the saturated fatty acids) in amounts which ranged between 15.5% and 18.6. The fatty acid compositions were changed moderately at different extraction conditions but the results showed no significant difference between using supercritical and near-critical CO_2 extractions [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

Another source of value for the wheat bran is its protein content. Once the oil has been removed from the bran, the relative amount of protein will obviously be increased. This makes possible to sell the defatted by product after the oil extraction as a protein-rich and low-fat animal feed or to extract this protein content to be sold as a value-added product [SPARKS et al., 2006].

2 Objective

The goal of this research is to extract wheat bran oil using a supercritical CO_2 extracting unit while establishing the best conditions in order to obtain higher yields of the extract. A lot of literature has been written about the extraction of oil out of cereal brans, especially from rice or rye [CHAO-RUI et al., 2008; CHIH-HUNG et al., 2008; DA CRUZ FRANCISCO et al., 2005; SARMENTO and FERREIRA, 2006; SPARKS et al., 2006], but this technology has not been so often applied for wheat bran.

The wheat bran extract is commonly a mixture of oil with other less valuable phases such as water, waxes and other compounds that need to be separated after the extraction. We pretend therefore to optimize the extraction in order to maximize the oil in the extract and reduce the amounts in the other phases in order to simplify the subsequent refining process of the oil. Another interesting aspect of cereal brans is their content in highly valuable antioxidants such as tocols, sterols, carotenoids or alkylresorcinols [KYUNG-TAE et al., 2010; KAMAL-ELDIN et al., 2009; KORYCINSKA et al., 2009], which increases the possibilities to find a commercial application for the product. Another goal of this research is therefore to maximize the amounts of these interesting compounds within the extracts by optimizing the extraction parameters and obtaining healthier and more valuable oil.

The importance of knowing the fatty acid profile of an oil sample locates in that it has direct implications in human health. The consumption of oils rich in saturated fatty acids has been associated with cardiovascular disease by organizations such as the World Health Organization, the American Dietetic Association, the World Heart Federation, the United States Food and Drug Administration, and the European Food Safety Authority. It is recommended instead to substitute its consumption with unsaturated and mainly polyunsaturated fat, which is also believed as well to reduce the risk of other diseases such as breast cancer [PATTERSON et al., 2010]. Diets that are deficient in essential fatty acids (EFA) such as linoleic (18:2), linolenic (18:3) and arachidonic acid (20:4), are a cause for growth retardment and the appearance of dermal symptoms [CHOWDHURY et al., 2007]. The third goal of this research is to determine this profile and see if it matches with the findings made by GO-WOON et al. (2010) and KYUNG-TAE et al. (2010) and to see whether the extraction conditions have a significant impact on it.

3 Materials and methods

3.1 Raw material

The CO_2 extractions performed in this research are done using wheat bran produced by Erste Wiener Walzmühle Vonwiller GmbH, from pure, mechanically cleaned wheat coming from the region of Lower Austria in Austria, bagged in 30 kg paper bags which, according to the supplier should be stored in a dry environment and has a minimum durability of 7 months after its delivery. The product has a loose texture, a smooth brown colour, free from insects or other contaminants and the flavour and taste is that one of a normal product, with no foreign odours or flavours.

According to the supplier, the bran has a minimum humidity of 11% and a maximum humidity of 15% when delivered, a minimum ash content of 6%, a minimum protein content of 14%, and a maximum crude fiber content of 12%. The nutritional values according to the supplier can be observed on Table 2. Microbiologically speaking, the requirements of the product are, according to the supplier, those present on Table 3.

Table 2: Nutritional values of the wheat bran according to the supplier [Erste Wiener Walzmühle Vonwiller GmbH].

	Per 100 g product
Energy [kJ]	1085
Energy [kcal]	262
Protein [g]	14.9
Carbohydrates [g]	17.3
Sugars [g]	3.2
Dietary Fiber [g]	45.4
Fat [g]	4.7
Saturated Fatty Acids [g]	0.7
Sodium [g]	0.002

Table 3: Microbiological specification according to the supplier [Erste Wiener Walzmühle Vonwiller GmbH].

Analyse	***Limit values***
Total count	500000CFU/g
Enterobacteriaceae	100000 CFU/g
E. Coli	10 CFU/g
Pathogens	Negative in 25g
Bacillus Cereus	<100 CFU/g
Yeasts	<2000 CFU/g
Molds	<10000 CFU/g

Two types of bran are used, a normal type and a treated one (see Figure 3). The second type, called “food bran” is given a steam treatment in order to deactivate certain enzymes such as lipases, which is supposed to reduce the hydrolysis of fatty acids and the consequent formation of free fatty acids in the fat extraction.

Figure 3: The two wheat bran sorts used in this research: Normal wheat bran (left) and food wheat bran (right).

3.2 Raw material pre-analysis

3.2.1 Dry substance – Air Oven Method

3.2.1.1 Principle

This method determines the moisture of a sample by measuring its loss of weight after drying it in the oven under specific conditions. It is a method that can be applied to flour, semolina, bread, all kinds of grains and cereal products, and other food products except those that are sugar coated.

3.2.1.2 Apparatus and chemicals

- Aluminium trays
- Weight balance, BP 210S, Sartorius, Germany
- Dry oven, 100-800, Memmert, Germany (see Figure 4)
- Desiccator

Figure 4: Dry oven used for the analysis.

3.2.1.3 Procedure

Every sample is analysed in double determination. First of all, weigh the aluminium trays and record the weight.

Weight in approximately 3 g of the bran sample into its aluminium dish. Subtract tare weights and record weight of sample. Place the dishes in a shelf in the dry oven at a temperature of 101 ºC for approximately 12 hours.

Remove the aluminium dishes from the oven and place them very rapidly inside a desiccator to allow them to cool down while avoiding the air moisture to be absorbed by the

now dehydrated sample. Leave the dishes in the desiccator for 45-60 minutes until they have reached room temperature and then weigh them on the balance to determine the dry matter and the loss of weight (moisture) of the sample.

3.2.1.4 Calculations

The total dry matter of the sample in g is calculated by subtracting the weight of the dish to the weight out of the sample after getting out of the oven:

$$\boldsymbol{DM = W_O - W_e}$$

Where:

- DM = dry matter [g]
- W_o = weight out (sample + tray after drying in the oven) [g]
- W_e = Weight of the empty dried aluminium tray [g]

The percentage of dry matter can be calculated as follows

$$\%\boldsymbol{DM} = \left(\frac{\boldsymbol{DM}}{\boldsymbol{W_i}}\right) \times \mathbf{100}$$

Where:

- %DM = percentage that the dry matter represents in the sample's total weight [wt/wt]
- DM = dry matter [g]
- W_i = weight in of the bran sample [g]

The percentage of moisture in the sample can be calculated will be the remaining percentage:

$$\%\boldsymbol{M} = \mathbf{100} - \%\boldsymbol{DM}$$

Where:

- %M = percentage that the humidity represents in the sample's total weight [wt/wt]
- %DM = percentage that the dry matter represents in the sample's total weight [wt/wt]

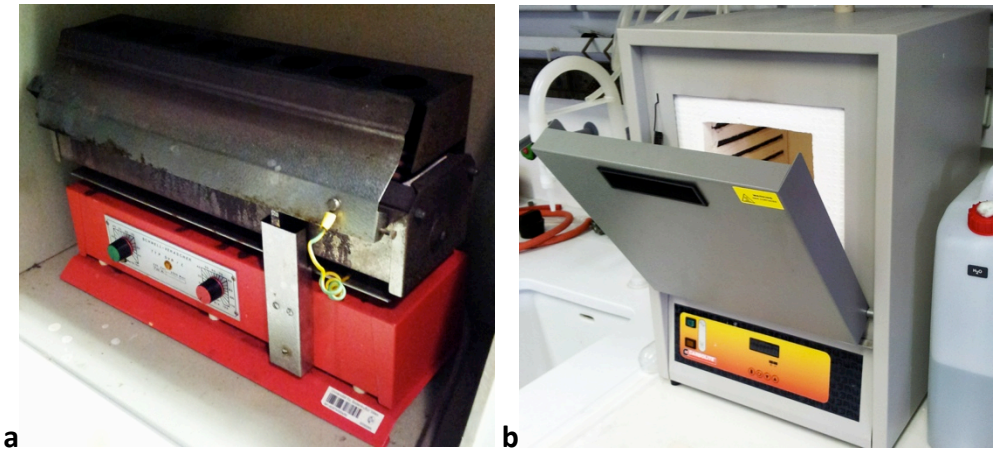
3.2.2 Ash – Basic method (AACC Method 08-01)

3.2.2.1 Principle

Dry ash procedures use a high temperature muffle furnace capable of reaching temperatures of around 650 ºC. Water and other volatile materials are vaporized and organic substances are burned in the presence of oxygen into CO_2, H_2O and N_2 leaving the sample in a state that it looks practically white [AACC, 2006].

3.2.2.2 Apparatus and chemicals

- Weight balance, BP 210S, Sartorius, Germany
- Porcelain crucibles
- Desiccator
- Muffle furnace, ELF 11/6B, Carbolite, England (see Figure 5b)
- Forceps
- Quick asher, TYP SVR/E, Harry Gestigkeit, Germany (see Figure 5a)
- H_2O_2, 3%



a b

Figure 5: Quick asher (a) and muffle furnace (b) used for this analysis.

3.2.2.3 Procedure

Every sample is analysed in double determination.

Introduce the porcelain crucible into the muffle furnace at a temperature of 650 ºC during a period of 2 hours. After that time, put the crucible into the desiccator allowing it to cool down to room temperature before weighing it on the balance.

Mill a portion of the bran and weigh in approximately 2 g into a porcelain crucible and use the quick asher to burn it. Remove the crucible of the quick asher once there're are no more fumes coming out of the sample.

Put the crucible into the muffle furnace at a temperature of 650 ºC ± 10 ºC and leave it there until the sample has acquired a white-greyish colour.

At this point, remove the crucible from the furnace and bring it to room temperature inside of the desiccator. Weigh the crucible on the balance.

3.2.2.4 Calculations

The concentration of ash in the sample can be calculated the following way:

$$\boldsymbol{c = \frac{W_o - W_e}{W_i} \times 100}$$

Where:

- c = concentration of ash in the sample [%]
- W_o = weight out with the crucible [g]
- W_e = empty weight of the crucible [g]
- W_i = weight in of the sample [g]

3.2.3 Protein – Kjedahl Method

3.2.3.1 Principle

In the Kjedahl procedure, proteins and other organic food components in a sample are digested with sulfuric acid in the presence of catalysts. The total organic nitrogen is converted to ammonium sulfate.

The digest is neutralized with alkali and distilled into a boric acid solution. The borate anions formed are titrated with standardized acid, which is converted to nitrogen in the sample. The result of the analysis represents the crude protein content of the food since nitrogen also comes from non-protein components (note that the Kjedahl method also measures nitrogen in any ammonia and ammonium sulfate).

3.2.3.2 Apparatus and chemicals

- Weight balance, BP 210S, Sartorius, Germany
- Digest tubes, 250 mL Büchi, Switzerland
- Digest system, K-437, Büchi, Switzerland (see Figure 6a)
- Glass marbles
- Kjedahl catalyst tablets (Hg/Se-free), Merck, Germany
- Erlenmeyer flasks, 250 mL
- Distillator, K-360, Büchi, Switzerland (see Figure 6b)

- Titrator, 775 Dosimat, Metronohm, Switzerland
- H_2SO_4
- Water
- NaOH 30%
- Boric acid, 2%
- Methylene blue/red indicator
- Sodium hydrogen carbonate
- HCl, 0.1 M

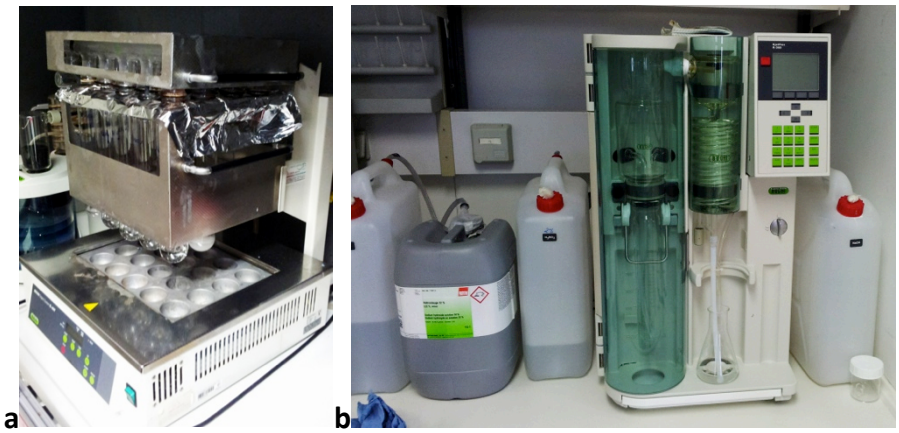


Figure 6: Digest system (a) and distillator (b) used in the experiments.

3.2.3.3 Procedure

Every sample is analysed in double determination.

Weigh approximately 1 g sample into the 250 mL digest tube and record the weight value. Add a 5 g Kjedahl tablet, then 10 mL H_2SO_4 and a glass marble into the tube. Introduce the tube into the digestor machine and close it safely. Start the digestion. During digestion, protein nitrogen is liberated to form ammonium ions, sulphuric acid oxidizes organic matter and combines with the formed ammonium and carbon and hydrogen elements are converted to carbon dioxide and water. Let the digestion run until the sample has acquired a greenish colour.

Allow the tubes to cool down for 5 minutes and then add between 10 and 20 mL of water approximately 10 – 20 mL water and cool the samples to cool down to room temperature. Add a layer of NaOH avoiding to agitate the mix. Connect the tube to the condenser with the tip of the condenser immersed in the flask and start the distillation using 20 mL of 2% boric acid solution. Give in 3-4 drops of methylene blue/red indicator into an Erlenmeyer and place it at the exit of the condenser.

Once the distillation is finished, take the Erlenmeyer in order to do the titration with standardized HCl 0.1 M of the borate anion, which is proportional to the amount of nitrogen in the sample. During titration, the solution changes quickly from green to blue and ends up in a green-brown colour. At this point, write down the amount of HCl used to do the titration, it will be necessary to calculate the amount of protein.

3.2.3.4 Calculations

The concentration of protein in the sample can be calculated the following way:

$$\boldsymbol{c = \left(\frac{H \times 0.1 \times 14.007 \times F_p}{W_i}\right) \times 100}$$

Where:

- c = concentration of protein in the sample [%]
- W_i = weight in of the sample [g]
- F_p = A factor is used to convert percentage of nitrogen to percentage of crude protein. Most proteins contain 16% nitrogen, so the conversion factor is:

$$\boldsymbol{F_p = \frac{100}{16} = 6.25}$$

3.2.4 Total Dietary Fiber (TDF) [AACC Method 32-05 and AOAC Method 985.29]

3.2.4.1 Principle

Dietary fiber is composed by a mixture of both hydrophilic organic compounds such as non-digestable oligosaccharides and soluble and insoluble polysaccharides as well as some partially hydrophobic compounds such as lignins, cutins and suberins.

The total dietary fibre (TDF) analysis is determined on duplicate samples that will be digested with several enzymes, filtered, dried and weighed before using one of the duplicates to determine the ash and the other one to determine the protein. The final TDF value is the result of subtracting the protein and ash weights to the weight of the residue.

3.2.4.2 Apparatus and chemicals

- Dispensers
  - 280 mL (for 95% ethanol)
  - 10 mL (for 78% ethanol, 95% ethanol and acetone)
  - 50 mL (for buffer)

- Beakers, 400 mL, tall-form
- Fritted crucibles
- Mill (0.3 - 0.5 mm)
- Aspirator, with regulator capable of regulating vacuum
- Boiling water bath (100 ºC)
- Shaking water bath (60 ºC)
- Weight balance, BP 210S, Sartorius, Germany
- Dry oven, UT6060, Heraeus Instruments, Germany (70 ºC)
- Timer
- Desiccator
- pH meter, Mettler Toledo, USA
- Spatulas
- Muffle furnace, 525 ºC, ELF 11/6B, Carbolite, England
- Aluminium foil
- Celite
- Heat stable α-amylase, Megazyme, Ireland
- Phosphate buffer (0.08 M; pH 6.0)
- NaOH (0.275 N)
- HCL solution (0.325 N)
- Ethanol
    - 95%
    - 78%
- Acetone
- Distilled water

3.2.4.3 Procedure

3.2.4.3.1 Preparation of the materials, buffers and solutions

In this experiment, the sample has to be previously dried and, in case the fat content is higher than 10%, it should be defatted as well. The total dietary fibre (TDF) analysis is determined on duplicate samples.

To prepare the fritted crucibles, ash overnight at 525 ºC in muffle furnace and remove Celite and ash material by using a vacuum. Soak in 2% Micro cleaning solution (reagent 7) at room temperature for 1 hour and rinse crucibles with water and deionised water. For final rinse, use 15 mL acetone and air dry. Add approximately 1.0 g Celite to dried crucibles and dry at 130 ºC to constant weight. Cool crucible in desiccator for approximately 1 hour and record weight of crucible containing Celite.

To prepare the phosphate buffer (0.08 M; pH 6.0), dissolve 1.4 g Na phosphate anhydrate and 9.68 g Na phosphate monobasic monohydrate in approximately 700 mL distilled water. Dilute to 1 L with water. Check pH with pH meter.

To prepare the sodium hydroxide solution (0.275 N), dissolve 11 g ACS grade NaOH in approximately 700 mL distilled water, using appropriate handling precautions, in 1 L volumetric flask. Cool and dilute to volume with water.

To prepare the Hydrochloric acid solution (0.325 N), dilute stock solution of known titer (i.e. 325 mL of 1.0 N HCl) to 1 L with water in volumetric flask.

3.2.4.3.2 Preparation of the sample

Use the previously described Air Oven method in order to determine the weight loss due to dehydration. Mill a portion of a dried sample to a 0.3 - 0.5 mm mesh. In case the fat content is higher than 10%, it should be previously defatted by using running a petroleum ether distillation three times with 25 mL portions per g of sample and the weight loss due to defatting should be recorded.

3.2.4.3.3 Method

A blank sample should be run together with the samples through the entire analysis in order to measure any contribution from the reagents to the final residue.

Weigh duplicate 1 g (±0.1 g) samples, into 400 mL beakers. The weights of the duplicates should not differ from each other in more than 20 mg. Add 50 mL of phosphate buffer (pH 6.0) to the samples and check their pH with the help of a pH meter. In case the pH differs from 6 (±0.1) this should be corrected.

Add 50 µL of a heat-stable α-amylase solution, cover the beakers with aluminium foil and place them in boiling water bath (100 ºC) for a period of 15 minutes gently shaking them every 5 minutes. Cool the samples to room temperature and adjust their pH to 7.5 (±0.1) by adding 10 mL of a 0.275 N NaOH solution checking their pH with the help of the pH meter.

Add 100 µL of a protease solution and cover the beakers with aluminium foil before incubating them at 60 ºC for a period of 30 min in a shaking water bath. After this time, cool the beakers to room temperature and add 10 mL of a 0.325 N HCl solution to adjust pH to 4.5 (±0.2) verifying it with the help of the pH meter.

Add 200 µL of amyloglucosidase and cover the beakers again with the aluminium foil before incubating them in a shaking water bath for 30 min at 60 ºC. Measure 280 mL volumes of 95% ethanol and preheat them at a temperature of 60 ºC before giving them into the samples. Allow the samples to precipitate at room temperature during an hour.

Use the balance to weigh crucibles containing Celite and distribute the content within by washing it with 78% ethanol. Apply suction to draw Celite onto the fritted glass as an even mat. Maintain the suction and quantitatively transfer the precipitates from the enzyme digests to the crucibles.

Wash and filtrate the residues with three successive 20 mL volumes of 78 % EtOH, two 10 mL volumes of 95 % ethanol, and two 10 mL volumes of acetone. In order to avoid the formation of gums during filtration, which would trap the liquid in the residue, break surface film with the help of a spatula. Dry the crucibles overnight in in the air oven at a temperature of 70 ºC and allow them to cool down to room temperature inside a desiccator afterwards. Use the balance to determine the weight of the residue by subtracting the crucible and Celite weights.

Use one of the duplicates to analyse its residue for protein by AACC Method 46 - 13, using N x 6.25 as conversion factor and incinerate the second duplicate for 5 hours at 525 ºC and cool inside of desiccator. Weigh and subtract the crucible and Celite weights to determine ash. A schematic diagram of the Total Dietary Fibre method can be seen at Figure 7.

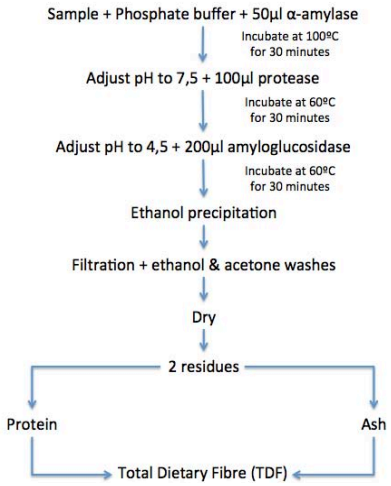


Figure 7: Analytical scheme for total dietary fibre procedure [MEGAZYME, 2011].

3.2.4.4 Calculations

The residue coming from the reagents is calculated the following way:

$$BPR = \frac{UABR \times P_b}{100}$$

$$\boldsymbol{BAR = \frac{UABR \times A_b}{100}}$$

$$\boldsymbol{CB = UABR - BPR - BAR}$$

Where:

- BPR = blank protein residue [mg]
- UABR = Av blank residue of duplicate blanks [mg]
- P_b = Protein in the blank [%]
- BAR = blank ash residue [mg]
- A_b = ash in the blank [%]
- CB = corrected blank [mg]

The residue in the sample is calculated the following way:

$$\boldsymbol{SPR = \frac{UASR \times P_s}{100}}$$

$$\boldsymbol{SAR = \frac{UASR \times A_s}{100}}$$

$$\boldsymbol{CSR = UASR - SPR - SAR - CB}$$

Where:

- SPR = sample protein residue [mg]
- UASR = Av sample residue of duplicate samples [mg]
- P_s = Protein in the sample [%]
- SAR = sample ash residue [mg]
- A_s = ash in the sample [%]
- CSR = corrected sample residue [mg]
- CB = corrected blank [mg]

To calculate the amount of total dietary fibre (TDF) in the sample:

$$TDF = \frac{100 \times CSR}{W_i} - M - F$$

Where:

- TDF = total dietary fiber [%]
- CSR = corrected sample residue [mg]
- W_i = weight in [mg]
- M = moisture [%]
- F = fat [%] (if the sample was defatted due to fat content higher than 10%)

3.2.5 Fat – Soxhlet method

3.2.5.1 Principle

Soxhlet extraction is a laboratory scalable method for the extraction of fats using petroleum ether as a solvent. Its goal is therefore to get to know the fat content of a sample. The solvent is heated, evaporated and condensed several times making it go through the sample, diluting and separating its fat content. Fat content is then measured by weight loss of the sample or by weight of the fat removed.

3.2.5.2 Apparatus and chemicals

- Dry oven, 100-800, Memmert, Germany
- Desiccator
- Spatula
- Weight balance, BP 210S, Sartorius, Germany
- Cotton, Rauscher, Austria
- Soxhlet equipment (see Figure 8) consisting of:
    - Porous extraction thimbles, VWR, Whatman, UK
    - Soxhlet condenser, Büchi, Switzerland
    - Soxhlet extractor, Büchi, Switzerland
    - Soxhlet clips
    - Round-bottomed glass flasks
    - Glass marbles
    - Heating mantle, 250 mL, Horst, Germany
- Petroleum ether, CP44.3, Roth, Germany

Figure 8: Soxhlet equipment used in this analysis.

3.2.5.3 Procedure

Every sample is analysed in double determination.

Introduce 3-5 glass marbles in the round-bottomed flasks and weigh them. Leave the flasks in the dry oven at a temperature of 105 ºC for a few hours. After that time, allow them to cool in a desiccator.

Weigh in about 10 g of the bran sample into a dry extraction thimble. The thimble has a porous surface allowing a rapid flow of petroleum ether during the extraction later on. Once the sample is inside, fill the remaining volume of the thimble with cotton. Weigh in the round-bottomed flasks.

Place the flask in the heating mantle, adjust the soxhlet extractor to its top with the help of a clip and introduce the thimble inside the extractor. Fill in 130 mL of petroleum ether through the top of the extractor, adjust the condenser to the top of the later one and secure it with a clip.

Turn on the air extractor and the cooling water cycle of the soxhlet. Start the heating mantle at level 3 (maximum) and once the solvent starts to boil inside of the flask, set it to level 1 (minimum). Let the cycle run for a period of 3 hours. After this period, allow the petroleum ether to evaporate once more and condense in the extractor until there is almost no solvent in the flask. Use the faucet in the extractor to remove the petroleum ether, which can be reused in further extractions. Turn of the mantle and disassemble the soxhlet.

Allow the thimble to cool down and dry by throwing it into a recipient inside of an air extractor and take the glass flasks to the dry oven once again where the remaining petroleum ether will be evaporated. After 1 hour, remove them from the oven and place them into the desiccator until they cool down. Weigh the flasks using the balance.

Clean the thoroughly the thimbles and turn of the air extractor.

3.2.5.4 Calculations

The concentration of fat in the sample can be calculated the following way:

$$\boldsymbol{c = \frac{(W_o - W_e)}{W_i} \times 100}$$

Where:

- c = fat content [%]
- W_o = weight out oft he flasks with the marbles and the fat [g]
- W_e = empty weight of the flasks with the marbles [g]
- W_i = weight in

3.3 Extraction process

3.3.1 Pre-treatment of the raw material (water content adjustment)

3.3.1.1 Principle

Water is added to the bran sample in order to obtain a mixture that will have a desired humidity that will influence the extraction yields. In case the original bran sample has a higher humidity than desired, the sample has to be left previously in a dry oven until the humidity is low enough, its new water concentration has to be analysed again with the dry oven method and then proceed with the water addition if it is needed.

3.3.1.2 Apparatus and chemicals

- Dry oven, UT6060, Heraeus, Germany
- Balance, Sartorius, Germany (0.1 mg accuracy)
- Beakers
- Mixer, RN10/VL 2, Varimixer, Denmark
- Spoon
- Plain water

3.3.1.3 Procedure

The amount of bran and water and bran to be mixed in order to obtain a mixture with a desired humidity value is calculated previously by using the values obtained in the previous dry matter analysis of the raw material as follows:

$$\boldsymbol{M = 100 - DM}$$

$$\boldsymbol{W_W = \frac{W_F}{100} \times (M_F - M)}$$

$$\boldsymbol{W_B = W_F - W_W}$$

Where:

- M = moisture of the sample [%]
- DM = dry matter of the sample [%]
- W_W = Water to be added to the mix [g]
- W_F = Desired final weight of the sample [g]
- M_F = Desired final moisture [%]
- W_B = Bran to be added to the mix [g]

In the eventual case the water content in the raw material is higher than the desired for the extraction, the sample has to be dried previously in a dry oven and then the dry

matter/humidity of the sample has to be measured once more in order to determine the amount of water to be added (if there is any). With the help of the beakers and the balance, measure the amount for each of both ingredients with accuracy.

Put the total amount of bran into the bowl of the mixer and turn on the machine at moderate revolutions to avoid the bran to be ejected of the bowl. While the machine is running, add the water drop by drop. After a few drops stop the machine and with the help of a spoon, scratch the rests of bran and water that are stuck to the base of the bowl and mix thoroughly before starting the machine again. Repeat the process until all the water is added and let the mixer work for an additional 10 minutes.

3.3.2 CO_2 extraction

3.3.2.1 Principle

The application of Supercritical Fluid Extraction (SFE) has grown steadily due to several advantages over classic organic solvent extractions.

CO_2 is the most widely used supercritical fluid because of its price, ease to obtain and properties (non-flammable, non-corrosive, non-toxic and inert to most materials). Moreover, it has are relatively low critical temperature (31.1 ºC) making it suitable for the extraction of heat sensitive bioactive compounds and reducing the energy costs within the process [GO-WOON et al., 2010; KYUNG-TAE et al., 2010]. Supercritical CO_2 extraction allows modifying the temperature and pressure within the process, which can improve its efficiency or increase the selective and yield of specific compounds.

3.3.2.2 Apparatus and chemicals

- Supercritical CO_2 extracting unit, 550 bar, R&D unit, Natex Prozesstechnologie, Austria (see Figure 9), consisting of:
  - a pump
  - an extractor (2 litre)
  - a separators
  - a computer with DigiVis software (see Figure 10)
- Glass balls, ≈10 mm Ø
- Beakers
- Weight balance, Sartorius, Germany
- CO_2 in gas form, UN 1013, Linde, Germany

Figure 9: CO_2 extracting unit used in these experiments.

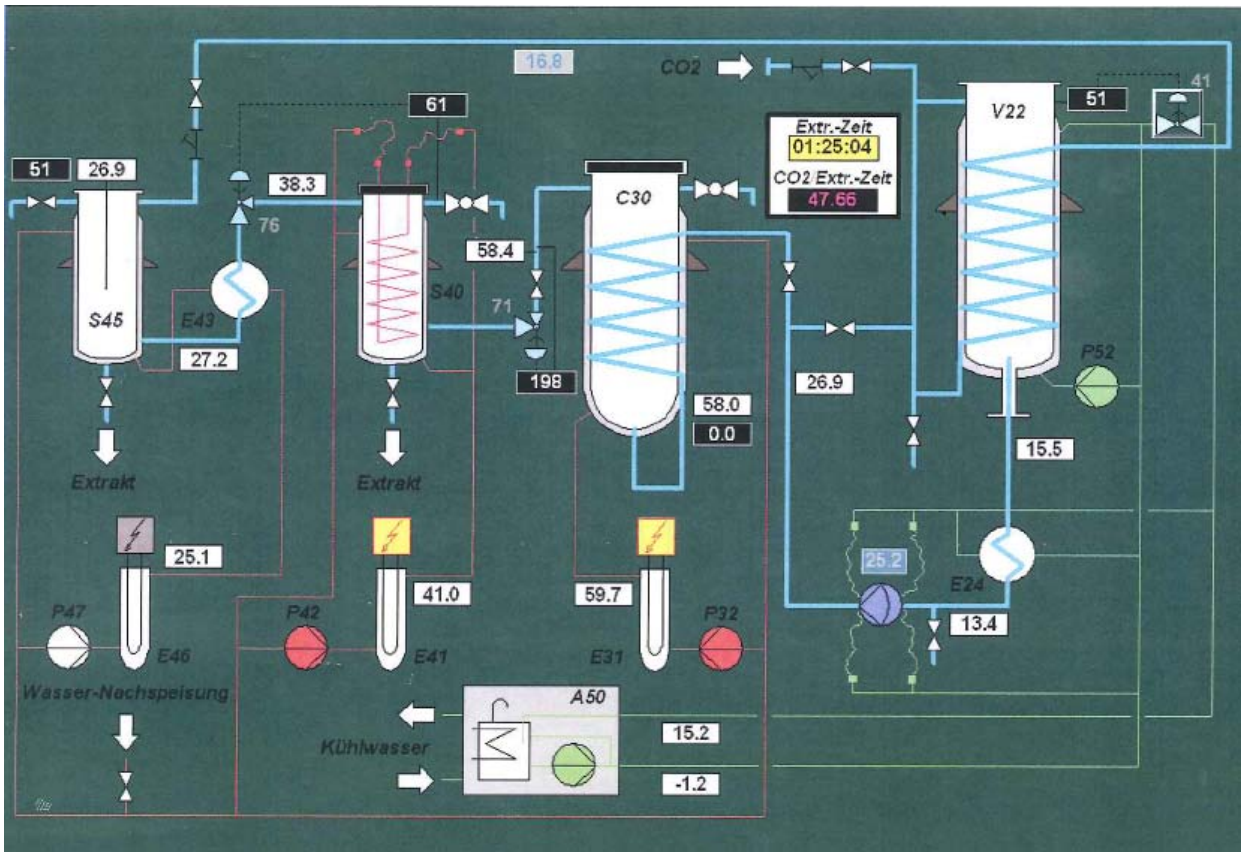


Figure 10: Schematic diagram of the supercritical CO_2 extracting unit used in this process.

Legend:

A50: Cooling unit	P42; P47: Pumps (heating units separators)
C30: Extractor	P52: Pump (cooling CO_2 storage vessel)
E24: Pre-cooler (heat exchanger)	S40, S45: Separators
E31: Heating units (extractor)	V22: CO_2 storage vessel
E41; E46: Heating units (separators)	___: Cooling cycle
E43: Evaporator (heat exchanger)	___: Heating cycle
P32: Pump (heating unit extractor)	___: CO_2 cycle

3.3.2.3 Procedure

3.3.2.3.1 Preparation of the machine

Switch on the power open the source and drain for the cooling water. Start the flow of compressed air, check that the cooling water temperature is 20 ºC and the pressure to 2-4 bar. Set the manometers at the following pressures:

- Manometer 1: 5 bar
- Manometer 2: 3.5 bar
- Manometer 4: 3.5 bar
- Manometer 4: 1.7 bar

Make sure the coolant stands between the level marks. Start the DigiVis software and turn on the pump P52, the coolant pump of the cooling unit (A50) as well as the cooling unit (A50) itself (see Figure 10). Start the heating unit of the extractor (P32) and the separator (P42) and set up the correct temperatures.

3.3.2.3.2 Preparation of the sample

The sample has to be mixed with glass balls before being introduced into the extraction cartridge of the extractor (C30). Their goal is to prevent the formation of channels and thus, a uniform density of the sample, to prevent clogging.

3.3.2.3.3 Filling the extractor

Before filling the extraction cartridge of the extractor with the mixture of bran and glass balls prepared in the previous point, it is to be assured that there exists no pressure

within it. In the case there is still pressure, which means that the extractor still contains CO_2 that needs to be slowly released by opening the valve 04V1.

Open the lid of the extractor by activation of the pneumatic valve nearby. And extract the cartridge. Unscrew the lid of the cartridge carefully avoiding damaging the seal and the worm screw. Fill in the extraction cartridge with the sample and place a perforated plate, a filter paper sheet, a sieve plate and a second perforated plate at their place at the top of the cartridge before closing the lid. This disposition of the previous items is important to avoid carryover of wheat bran from the extraction cartridge into the system. Introduce the cartridge into the extractor and close the lid.

The extractor needs now to be ventilated, which will be done by following several steps that will gradually and slowly increase and reduce the pressure within the extractor several times before the final value is achieved. The reason for this to be done is to avoid air coming into the system. Open the valve 03V3 that will fill in the extractor with CO_2 until a pressure of 10 bar is achieved. Extract the air by opening the drainage valve C30V4 until a pressure of 5 bars is reached. Inject CO_2 once more until 15 bars are reached and release air until the pressure is reduced to 10 bars. Fill in for a last time with CO_2 until the pressure of 20 bars is achieved and then reduce the pressure to 15 bars by ejecting air.

3.3.2.3.4 Extraction

Slowly open the valve 03V3 and compensate the pressure in the whole system by opening the valve between the separators a 70% and that one after the extractor a 20%.

Check that the by-pass valve 03V2 is open and turn on the pump P20 at a frequency 10 Hz then close the valve again. Increase the pressure in the system paying attention that the CO_2 flow rate does not go over 25 kg/h and that the difference of pressure of the extractor does not exceed ±5 bars. Do so by increasing the frequency of the pump until the desired pressure is achieved then gradually close the valve after the separator until the desired pressure is achieved. Change then from manual to automatic operation. Progressively close the valve after the extractor until the pressure is achieved and change the operation of the valves from manual to automatic, which will allow the system to maintain the pressure at the desired value.

Measure the weight of the empty beaker with the help of the balance and place it at the exit of the separator. Sampling is done every 20 minutes by opening the valve S40V4 of the separator. The time to close again the valve comes when dry ice is the only thing coming out of the separator. Repeat the same procedure several times until no more extract is coming out. Once the extraction lies in the beaker, it is to be taken to the balance in order to determine its weight by subtracting the weight of the beaker measured previously.

After reaching the end of the extraction, the shutdown of the system begins, where the CO_2 flow rate as well as the pressure difference between the extractors should be watched out closely. Reduce the frequency rate of the pump in 3 steps and the pressure in the valve 05C3000Z between the extractor and the separator slowly in 20 steps. Set that valve at automatic operation so that the system takes care of maintaining the new pressure value. At the point when the pressure has reached 130 bar and the pump is working at 10 Hz, switch again to manual operation. Open the by-pass valve 03V2, close the valve 03V3 situated before the extractor and then turn of the pump P20 (see Figure 10). Compensate the pressure at the valves between separators and between separator and extractor to approximately 50 bars and close the valves afterwards. Turn off the heating circuit of the separator S40 and reduce the pressure within the separator by opening slowly the drainage valve S40V4. Before unscrewing the lid of the separator in order to clean the inner walls and spiral, activate the proximity sensor.

Close the separator and inject CO_2 inside of it by slightly opening the valve 05C3000Z between extractor and separator until a pressure of 10 bars is reached, then close the valve back again. Open the exhaust valve S40V3 until the pressure has fallen to 5 bars. This will displace the air within the separator and push it out of the system. Repeat the previous process this time from 15 to 10 bars and again from 20 to 15 bars. Compensate the pressure between separator and extractor by opening the valve 05C3000Z and then close the valve. Open the exhaust valve 04V1 in order to remove the gas within the extractor. Before proceeding to open the lid of the extractor, activate the proximity sensor and once the lid is removed, take out the cartridge from the extractor and empty it over a sieve standing over a tared recipient in order to measure the weight of the by-product. The sieve is used to separate the bran-looking by-product from the glass balls that were with the bran inside the cartridge. Make sure to remove all glass balls before recording the weight. Wash the inner walls of the extractor as well as the cartridge and close it back again.

3.3.2.3.5 Cleaning

After the last extraction, it is necessary to run a cleaning in the whole extraction system by filling the extractor with approximately 300 mL of ethanol and following the normal procedure described in the previous points. The conditions in the system at which the cleaning will take place are:

- Extractor: 200 bar, 60 ºC
- Separator: 60 bar, 40 ºC

After all the steps are completed, extract the alcohol of the separators 1 and 2, switch off the system, the pressure air and the cooling water source and drain.

3.3.2.4 *Calculations*

The weight loss of the bran during the extraction in calculated the following way:

$$\boldsymbol{W_l = W_i - W_{bp}}$$

Where:

- W_l = weight loss of the bran during the extraction [g]
- W_i = weight in of the bran into the extractor [g]
- W_{bp} = weight out of the by-product [g]

The losses in the extract are calculated the following way:

$$\boldsymbol{L = \frac{W_l - W_e}{W_l} \times 100}$$

Where:

- L = losses of extract occurred during the process [%]
- W_l = weight loss of the bran during the extraction [g]
- W_e = weight out of the extract [g]

3.4 Oil sample preparation

3.4.1 Centrifugation

3.4.1.1 Principle

Wheat bran extracts coming out of a CO_2 extracting unit do it in a semi-solid form due in part to its high contents of waxes, and at very low temperatures due to the nature of the extraction process (see Figure 15). Together with the oil, they contain high amounts of water, waxes and other compounds, which makes the physical separation necessary.

Centrifugation is an easy laboratory-scalable method for separation of compounds. When subjected to centrifugal forces, the different compounds in a fluid separate depending to their mass. A centrifuge works on the principle of increasing effective gravity (g's) to accelerate the rate of settling of the different suspended compounds more dense than the buffer. Effective gravity increases as the radius (distance between the axis of the centrifuge and the sample) does , or with the square of the rate of rotation. This way:

$$\boldsymbol{F = m \times r \times R^2}$$

Where:

- F = force
- m = mass
- r = radius
- R = rotation rate

In other words, doubling rotation speed increases 4 times the g's.

After centrifuging, the liquid is called "supernatant" and the solids at the bottom of the tube are called "pellet".

3.4.1.2 Apparatus and chemicals

- Hot water bath, D8, Haake, Germany
- Essay tubes, 50 mL, with cap, Falcon, Germany
- Weight balance, BP 210S, Sartorius, Germany
- Centrifuge, capable of achieving 3000 rpm, 5810R, Eppendorf, Germany
- Water

3.4.1.3 Procedure

Set the hot water bath at 40 ºC and introduce the samples (in their containers) into it until their solid part has become one with the rest of the solution. Empty their content into 50 mL Falco tubes and put them into the centrifuge machine. To avoid danger of accidents during centrifugation and damage for the equipment, it is necessary that the weights at each side of the axis are equal up to ±0.1 mg. This is achieved by introducing extra tubes filled in with water to compensate the weight at the other side of the axis.

The centrifuge is set up at 3000 rpm during 10 minutes and the tubes with the samples are extracted of the machine after this time. At this point there should be 3 clear different phases in the tubes. The separated oil should remain at the top followed by a solid phase comprising waxes and other compounds and finally a water phase.

3.4.2 Manual separation

3.4.2.1 Principle

The sample is disposed in several phase-layers after centrifugation, and those are to be separated in order to obtain refined compounds.

3.4.2.2 Apparatus and chemicals

- Essay tubes, 20 mL, with cap, Falcon, Germany
- Pasteur pipettes
- Spatula
- Weight balance, BP 210S, Sartorius, Germany

3.4.2.3 Procedure

The upper phase of oil of each sample is removed carefully with the use of Pasteur pipettes and put into 20 mL essay tubes (see Figure 11). A second and a third tube are used for each sample, one containing the intermediate waxy phase, which will be removed out of the tube coming out of the centrifuge with the help of a spatula, and another one to contain the bottom water phase of the centrifugation extract. The empty weight of the tubes can be previously measured with the help of a weight balance to know the amount in weight that every phase represents in each sample.

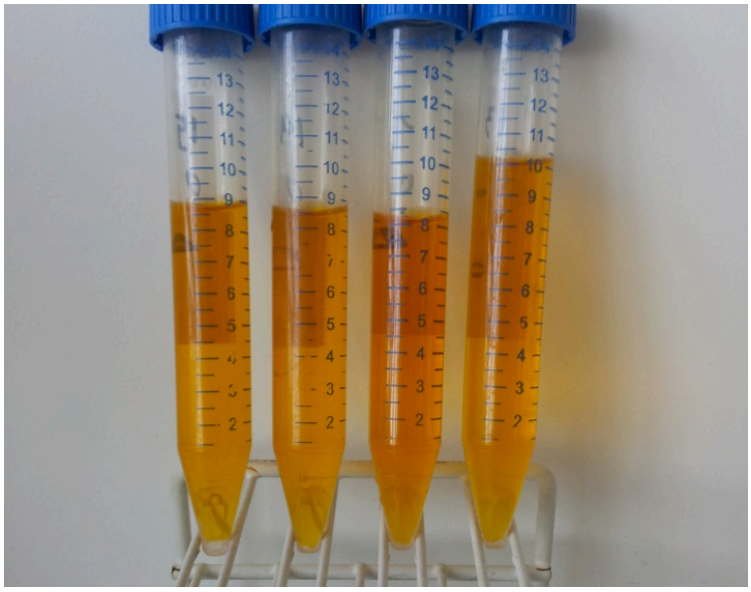


Figure 11: Wheat bran oil after its centrifugation and manual separation.

3.4.2.4 Calculations

Concentration of the different components (oil, water or waxes) in the extraction are calculated as follows:

$$c = \frac{W_p}{W_o} \times 100$$

Where:

- c = concentration of the component (oil, water or waxes) [%]
- W_p = weight of the phase after manual separation [g]
- W_o = weight out of the extract after CO_2 extraction [g]

3.5 Chemical analysis of the oils

3.5.1 Acid Value [IUPAC Method 2.201]

3.5.1.1 Principle

The acid value or acid number of the sample specifies how much KOH (in mg) needs to be used to neutralise the free fatty acids in 1 g of fat. It is used to quantify the amount of acid present in an oil sample.

The acidity of an oil sample can be caused by the formation of free fatty acids due to hydrolysis of triglycerides, which is a sign of oil deterioration.

3.5.1.2 Apparatus and chemicals

- Erlenmeyer flasks, 250 mL
- Burette, 25 mL
- Ethanol, 95%
- Diethyl ether
- Potassium hydroxide
- Phenolphthalein

3.5.1.3 Procedure

Prepare a 10 g/l phenolphthalein solution in ethanol and another one of potassium hydroxide in ethanol 0.1 M. Prepare a solution of diethylether and ethanol 1:1 and add 0.3 mL of the phenolphthalein solution per 100 mL solution. Use KOH to neutralise it.

Weigh the sample into an Erlenmeyer flask under the conditions stated at Table 4.

Table 4: Amount of sample to be weighed in depending on the expected acid value [IUPAC, 1987].

Expected acid value	*Weigh in of the sample [g]*	*Precision of the weighing [g]*
<1	20	0.05
1 - 4	10	0.02
4 - 15	2.5	0.01
15 - 75	0.5	0.001
>75	0.1	0.0002

Dilute the sample by adding 150 mL of the prepared solution into the Erlenmeyer flask. Titrate the sample using the 0.1 M potassium hydroxide standard solution. The phenolphthalein present in the solution will act as an indicator. In possible case of a standard solution usage superior to 20 mL during titration, repeat the process using a 0.5 M solution.

3.5.1.4 Calculations

The acid value of the sample specifies how much KOH (in mg) needs to be used to neutralise the free fatty acids in 1 g of fat. It is calculated as follows:

$$\boldsymbol{AV = \frac{56.1 \times C_{st} \times V_{KOH}}{W_i}}$$

Where:

- AV = acid value [g KOH/g fat]
- C_{st} = concentration of the standard solution [M]
- V_{KOH} = amount of KOH used in the titration [mL]
- W_i = weight in of the sample [g]

The acidity is calculated as follows:

$$\boldsymbol{A = \frac{C_{st} \times 282 \times V_{KOH}}{10 \times W_i}}$$

Where:

- A = acidity [%]
- C_{st} = concentration of the standard solution [%]
- V_{KOH} = Amount of KOH used in the titration [M]
- W_i = weight in of the sample [g]

3.5.2 Thin-Layer Chromatography

3.5.2.1 Principle

Thin-Layer Chromatography (TLC) is a chromatographic method based on the principle of separation, which depends on the relative affinity of the different compounds towards both a stationary and a mobile phase. The compounds travel over the surface of the stationary phase driven by the mobile phase (solvent) thanks to the effect capillary action.

Those compounds with higher affinity to the stationary phase move slowly over its surface whilst those with less affinity travel faster while the others travel faster and this is the way separation is achieved. After their separation, compounds are visualized over the surface as individual spots.

TLC is a good method to get a picture of the amount of free fatty acids, triglycerides, diglycerides and other compounds such as waxes present in an oil sample.

3.5.2.2 Apparatus and chemicals

- Ready-made TLC plates with stationary phase
- TLC glass container with lid
- Glass beakers (different volumes)
- Pipettes (different volumes)
- Forceps
- Desiccator
- Spray diffuser made of glass
- Air oven, Heraeus Oven, Thermo scientific, Germany (120 ºC)
- n-Hexane
- Diethyl ether
- Acetic acid
- Methanol
- Sulphuric acid

3.5.2.3 Procedure

Prepare a dilution of the oil sample into n-Hexane (1/10 or 1/100). With the help of a pipette, extract and place a drop of the dilution at the bottom of the TLC plate. In case several samples are going to be measured at the same time, make sure all of the drops are placed in the plate at the same height in the plate, to facilitate the subsequent comparison. Avoid touching the plate with bare fingers since these will leave oil rests into the plate that could ultimately alter the visible results of the TLC. This can be done using a forceps.

In a glass beaker prepare the mobile phase, consisting of 84% n-hexane, 15% diethyl ether and 1% acetic acid. Tip out a small amount of the mobile phase into the TLC glass container in a manner that the whole bottom of the container is covered by a thin layer of the solution. Place the TLC plate vertically in the container making sure the bottom of it is in contact with the mobile phase layer at the bottom of the container. Wait for several minutes until the mobile phase has almost reached the top of the plate due to the visible effects of capillarity and then extract the plate out of the container with care using the forceps and place it into a desiccator until the mobile phase solution dries off.

Prepare a solution of 10% sulfuric acid and 90% methanol and empty it into the spray diffusor. Spray the solution with care over the TLC plate making sure every part of it gets sprayed. Place the plate into the dry oven set up at 120 ºC for around 20 minutes.

3.5.3 Fatty Acids Methyl Esters (FAME)

3.5.3.1 Principle

The Fatty Acids Methyl Esters analysis is one of the most common procedures performed in the analysis of lipids and it helps to determine the fatty acid profile of an oil sample. It consists in the preparation of the methyl ester derivatives of fatty acids before an analysis is carried out with the help of a gas chromatograph. The fatty acid composition of an oil sample is interesting in the measure that it has implications in human health since oils represent up to 40% of the energy intake in western diets.

Gas chromatography (GC), like for all other chromatographic techniques, requires of a mobile and a stationary phase. The mobile phase, also called carrier gas is an inert gas (helium, argon, nitrogen, etc.), whilst the stationary phase consists of a capillary column of small diameter coated with the liquid stationary phase. Different compounds are separated because of their interaction with the stationary phase. A compound with a stronger interaction will remain for a longer time attached to the stationary phase and therefore it will take longer for this compound to go through the column. This is what is called retention time. GC with flame-ionization detection (FID) is the most used technique for the determination of fatty acid compositions, after they are converted to their ester derivatives. The technology is already mature and its functioning is well known, and allows experimental measures with high precision. GC can identify individual fatty acids with reasonable certainty from their relative retention times, especially when the analysis is carried out with a variety of stationary phases, and taking into account the amount of knowledge that now has been accumulated with the passing of time on the compositions of all sorts of analytical samples [GUNSTONE et al., 2007].

3.5.3.2 Apparatus and chemicals

- Spatula
- Weight balance, ATL 224, Acculab, Germany (±0.001 g)
- Pyrex tubes with cap
- Volumetric pipette (2 mL)
- Measuring pipettes (10 mL)
- Pasteur pipettes
- Autosamplervials with cap

- Hot water bath (70 ºC)
- Centrifuge, SuperVario-N, Funke Gerber, Germany (>1100 rpm)
- Gas chromatograph, Thermo Scientific trace GC Ultra (see Figure 12) with:
    - Flame ionization detector (FID)
    - Split/splitless injector
    - Autosampler
- Internal standard (C17:0 methyl ester in Toluene, ca. 1 mg/mL)
- Methanol/HCl (prepared by adding 10 vol. f acetylchloride dropwise to 100 vol.of methanol)
- Nitrogen gas
- Potassium carbonate solution, wt/vol. (K_2CO_3) = 6%
- Sodium sulphate (water free)

Figure 12: Gas chromatograph used in this experiment.

3.5.3.3 Procedure

Every sample is analysed in double determination using margaric acid (C17:0) as an internal standard. The settings were as follows:

- Hydrogen flow: 2 mL/min
- Split ratio: 1-50 mL/min
- Detector: 250 ºC
- Injector: 250 ºC
- Temperature program:
    - 170 ºC for 1 min
    - 2,5 ºC/min up to 220 ºC
    - 220 ºC for 4 min

Weight in 10-20 mg of the oil sample inside the pyrex tubes and pipette in 2 mL of the internal standard (the amount of standard should be around 10% the amount of the

sample). Pipette in 3 mL of methanol or HCl. Displace the air within the tubes with nitrogen and close the tubes with their caps firmly right afterwards.

Put the tubes into a hot water bath at 70 ºC for transmethylation, leave them for a period of two hours shaking them often and allow them to cool down afterwards. Pipette in 5 mL of 6% Potassium Carbonate solution and take the tubes for centrifugation at 1100 rpm during 5 minutes.

With the help of a spatula, add a spatula-full of water-free sodium sulphate. Remove the upper organic part with the help of a Pasteur pipette. Shake the tube until the sample will be clear and fill in the autosamplervials with the clear part of the content of each tube. Proceed to analysis with the Gas Chromatograph.

3.5.3.4 Calculations

The percentage of fatty acid is calculated as follows:

$$\% \, \boldsymbol{FAME} = \boldsymbol{A_{FA}} \div \boldsymbol{A_{Tot}} \times \mathbf{100}$$

Where:

- A_{FA} = area of the peak of the fatty acid in the chromatogram
- A_{Tot} = sum of the areas of the fatty acids

The concentration of a fatty acid is calculated as follows:

$$\boldsymbol{c} = \frac{\dfrac{\boldsymbol{A} \times \boldsymbol{W_{IS}}}{\boldsymbol{A_{IS}}}}{\boldsymbol{W_i}} \times \mathbf{100}$$

Where:

- c = concentration of a fatty acid [g FAME/100 g total fat]
- A = area of the peak of a fatty acid in the chromatogram
- W_{IS} = weight in of the Internal Standard
- A_{IS} = area Internal Standard
- W_i = weight in of the sample

3.5.4 Sterols (Gas Chromatography)

3.5.4.1 Principle

Phytosterols, phytostanols and their esters are a group of steroid alcohols and esters that naturally occur in plants. Gas chromatography is the most broadly used tool for the chromatographic separation, identification and quantification of plant sterols (phytosterols). There are many standardized methods available but all of them consist in the same steps: sample weighing, addition of internal standard, acid and/or alkaline hydrolysis, extraction of unsaponifiables, derivatization, and chromatographic analysis.

Gas chromatography, like for all other chromatographic techniques, requires of a mobile and a stationary phase. The mobile phase, also called carrier gas is an inert gas (helium, argon, nitrogen, etc.), whilst the stationary phase consists of a packed column of small diameter coated with the liquid stationary phase. Different compounds are separated because of their interaction with the stationary phase. A compound with a stronger interaction will remain for a longer time attached to the stationary phase and therefore it will take longer for this compound to go through the column. This is what is called retention time.

3.5.4.2 Apparatus and chemicals

- Weight balance, ATL 224, Acculab, Germany (±0.001 g)
- Screw-capped tubes
- Hot water bath
- Gas chromatograph, Thermo Scientific trace GC Ultra (see Figure 12) with:
    - Flame ionization detector (FID)
    - Split/splitless injector
    - Autosampler
- Dry oven, Pantatherm D, Salvis, Germany
- Alpha-cholestane (1 mg/mL in hexane)
- Potassium hydroxide (600 g/l)
- Ethanol (95%)
- Sodium chloride (10 g/l)
- Ethanolic pyrogallol (60 g/l)
- Sodium chloride (10 g/)
- n-Hexane
- Ethyl acetate
- Pyridine

- BSTFA + TMCS solution, 99:1, Sylon BFT, Supelco, USA

3.5.4.3 Procedure

Every sample is analysed in double determination. The settings were as follows:

- Carrier gas: 90 KPa
- Split injection: 100 mL/min
- Detector: 300 ºC
- Injector: 300 ºC
- Temperature program:
  - 100 ºC for 2 min
  - 30 ºC/min up to 270 ºC
  - 3 ºC/min up to 290 ºC

Weigh in approximately 0.5 g of oil sample into a screw-capped tube using a balance. Give into the tube 2 mL of potassium hydroxide 2 mL of ethanol, 2 mL of sodium chloride, and 5 mL of ethanolic pyrogallol, which is added as antioxidant.

Place the tube in a hot water bath at a temperature of 70 ºC for 45 minutes to allow the alkaline digestion to happen, making sure to shake it every 5-10 minutes. After this time, rapidly cool the tube in an ice bath and add 15 mL of sodium chloride.

Prepare a portion of n-hexane and ethyl acetate (9:1 v/v) and use 15 mL to extract the suspension twice collecting the organic layer first and evaporating then to dryness.

Add 100 µL pyridine and 100 µL BSTFA/TMCS solution and leave it for 30 minutes in a dry oven set at a temperature of 60 ºC until the solution is totally dry. Dissolve by adding 1 mL n-Hexane and inject into the Gas Chromatograph.

3.5.4.4 Calculations

The different compounds are identified on the gas chromatography – mass spectrometry system operated with the same column.

Using their area in the chromatogram, their individual concentrations in mg per 100 g oil can be calculated as follows:

$$c = \left(\frac{A}{\frac{A_{IS} \times 1.08}{W_i}} \right) \times 100$$

Where:

- c = concentration of the component [mg/100 g oil]
- A = area of the peak of a sterol in the chromatogram [.1*uV*sec]
- A_{IS} = area Internal Standard [.1*uV*sec]
- W_i = weight in [g]

3.5.5 Tocols (High Pressure Liquid Chromatography)

3.5.5.1 Principle

High Pressure Liquid Chromatography (HPLC), like other types of chromatography is based on a stationary phase that retains with more or less intensity the different components of a sample allowing their separation, and a mobile phase that transports these components through the stationary phase. In HPLC, the liquid solvent (mobile phase) is forced through the column by pressures up to 400 atmospheres, making the separation much faster. HPLC provides a convenient method for quantification purposes of tocols. Reverse phase columns are generally believed to have better stability and more durability. Normal phase columns, despite their disadvantage, are more efficient in the separation of β and γ isomers of tocopherols and tocotrienols. Normal phase analyses also provide the possibility of using organic solvents, which dissolve lipids much more efficiently [PANFILI, G. et al., 2003].

3.5.5.2 Apparatus and chemicals

- Weight balance, ATL 224, Acculab, Germany (±0.001 g)
- 25 mL volumetric flasks
- Ultrasound water bath
- High Pressure Liquid Chromatograph (see Figure 13), normal phase, LC-9A, Shimadzu, Japan, counting with:
    - System controller, SCL-6B, Shimadzu, Japan
    - Auto injector, SIL-6B, Shimadzu, Japan
    - Column oven, CTO-6A, Shimadzu, Japan
    - Refractive index detector, RID-6A, Shimadzu, Japan
    - Fluorescence HPLC monitor, RF-535, Shimadzu, Japan
    - UV spectrophotometric detector, SPD-6A, Shimadzu, Japan
    - Photodiode array UV-VIS detector, SPD-M6A, Shimadzu, Japan
- N-Hexan
- Sodium sulfate

Figure 13: High Pressure Liquid Chromatograph used in the analysis.

3.5.5.3 Procedure

Introduce 1 g (±0.001 g) sample in a 25 mL volumetric flask. Fill in up until the mark with n-Hexan. Allow diluting in an ultrasound water bath. Proceed to HPLC analysis. The value for the area of the peaks has to be within the range of values obtained for the standard solutions. If this were not the case, proceed to dilute the sample with n-Hexan and repeat the measurement.

3.5.5.4 Calculations

Using the area of the peaks in the chromatogram (see Figure 14), their individual concentrations in mg per 100 g oil can be calculated as follows:

$$\boldsymbol{c = \frac{(S \times A + R)}{\frac{1000 \times V}{W_i \times 100}} \times DF}$$

Where:

- c = concentration of the component [mg/100 g]
- S = slope calibration curve
- A = area of the peak of a tocol in the chromatogram
- R = distance between axis label and axis for the calibration curve
- V = volume used in the dilution [mL]
- W_i = weight in [g]
- DF = Dilution Factor

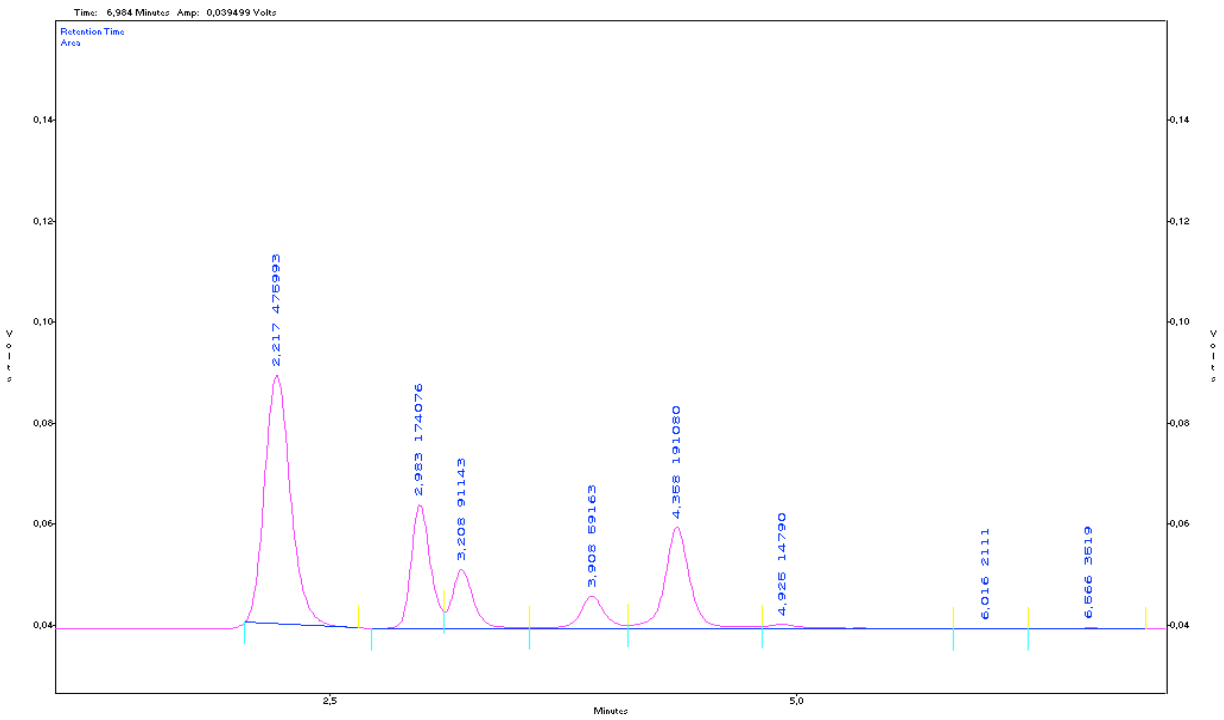


Figure 14: High pressure liquid chromatogram for tocols of a wheat bran oil sample.

3.6 By-product analysis

The Air Oven, Kjedahl and Soxhlet methods were used to determine the moisture, protein content and fat content respectively of the by product samples. The materials and methods used were the same as for the raw material pre-analysis (see points 3.2.1, 3.2.3, and 3.2.5).

4 Results and conclusions

4.1 Raw material pre-analysis

According to the supplier, the bran has a minimum humidity of 11% and a maximum humidity of 15% when delivered, a minimum ash content of 6% and a minimum protein content of 14%. Additionally, the fat content is supposed to be of 4.7 g per 100 g sample and the content of dietary fibre 45.4 g per 100 g sample.

4.1.1 Dry Substance – Air Oven Method

The results of our pre-analysis showed that the humidity was for the normal bran way under the value stipulated by the supplier (8.8% contrasting to the 11-15% assured). This could be a result of the storage conditions at our facilities, but still seems very unlikely that the humidity in our bran could be reduced by more than 2% in a room with no special environmental conditions of humidity and temperature. The values found at the food bran were within the range (see Table 5).

4.1.2 Ash – Basic Method [AACC Method 08-01]

The ash analysis revealed that the values given by our supplier deferred from those obtained at our analysis since the supplier specified that its bran had a minimum ash content of 6% and our analysis gave a result of 5.3%. This could possibly be to the supplier using a different method for its determinations.

4.1.3 Protein – Kjedahl Method

Proteins, perform various biological functions in the human body and differ from one another because of their chain or chains of aminoacids. Humans, like other animals, cannot biosynthesise essential amino acids as most microorganisms and plants do. Amino acids are obtained through the ingestion and digestion of foods containing protein. The acid environment in the stomach denaturates the protein and the action of certain enzymes (proteases) breaks it down into amino acids. That is the reason why it is very important for us to obtain them through the diet. According to the supplier, the minimum protein value in their product was 14%. Our findings showed that the bran we received had protein values that were slightly over 16%.

4.1.4 Total Dietary Fiber (TDF) [AACC Method 32-05 and AOAC Method 985.29]

Dietary fiber is composed by a mixture of both hydrophilic organic compounds such as non-digestable oligosaccharides and soluble and insoluble polysaccharides as well as some

partially hydrophobic compounds such as lignins, cutins and suberins. The Total Dietary Fiber value is of interest due to the nutritional properties of fibre and its impact in human health. Consumption of diets rich in dietary fiber has been often hypothesized to lower the risk of coronary heart disease, diabetes, and some cancers. Some studies have linked the total dietary fiber intake with the cause of specific death.

PARK et al. (2011), during a follow up that lasted 9 years, identified a total of 20126 deaths in men and 11330 in women. Dietary fiber intake was associated with a significantly lowered risk of total death and with a lowering in the risk of death from cardiovascular, infectious, and respiratory diseases by 24% to 56% in men and by 34% to 59% in women.

According to the American Food and Drug Administration, diets containing certain amounts of soluble fibres coming from cereals such a beta-glucans, reduce the risk of coronary heart disease [ANONYM, 2013].

The specification sheet of the supplier provided with the product implied that the total dietary fibre value for the bran was of 45.4%. Our analysis differed at this point as well, since according to our findings the value was over 48%.

4.1.5 Fat – Soxhlet Method

The fat content was supposed to be 4.7% and our analysis showed this value was inferior for both the normal (4.3%) and the food bran (2.4%). Significantly lower was the value for this last one and this could be a result to this one coming from a different harvest at a different moment of the year (i.e. wheat cultivated during winter is supposed to contain a higher amount of water and lower amount of fat) or to a different variety of wheat.

Table 5: Results of the raw material analysis of the wheat bran.

	Normal bran	***Food bran***	***Values according to supplier specification sheet***
Water content [%]	8.8	11.5	11-15
Dry matter [%]	91.2	88.5	85-89
Ash [%]	5.3	n.a.	>6
Protein [%]	16.3	n.a.	>14
Total Dietary Fiber [%]	48.2	n.a.	45.4
…as a part of the total dry matter [%]	52.9	n.a.	n.a.
Fat [%]	4.3	2.4	4.7

4.2 Extraction

In this research we ran experiments with different pressures and temperatures in order to determine the best possible extraction conditions to increase the yields of oil and the concentration of bioactive compounds in the extraction, and to compare our results to existing literature. Pressure and temperature are the commonly modified parameters in a supercritical CO_2 extraction. Additionally, we wanted to study a third parameter, which is usually not analysed in researches: the moisture (see Table 6).

Table 6: Experimental plan followed for the supercritical CO_2 extractions in this research.

Sample	*Bran type*	*Water content [%]*	*Pressure [bar]*	*Temperature [ºC]*	*Weight in [g]*
1	Normal	11	450	40	500
2	Normal	9	350	40	500
3	Normal	11	350	50	500
4	Normal	13	450	50	500
5	Normal	13	350	60	500
6	Normal	11	450	60	500
7	Normal	9	250	50	500
8	Normal	9	450	50	500
9	Normal	13	250	50	500
10	Normal	11	350	50	500
11	Normal	9	350	60	500
12	Normal	11	250	40	500
13	Normal	11	250	60	500
14	Normal	11	350	50	500
15	Normal	13	350	40	500
16	Normal	13	450	60	500
17	Food	13	450	60	350
18	Normal	12.7	450	60	500
19	Food	12.7	450	60	350
20	Normal	9	449.9	40	500
21	Food	9	449.9	40	350

The reason a different weight in was used for the food bran extractions was that this raw material has a lower bulk weight, meaning that it was impossible to fit 500 g of this bran into the cartridge of the extractor (see Table 6).

We worked with 3 different humidity values at first instance (9%, 11%, and 13%), modifying this parameter later on in further extractions according to the results obtained from the first extractions after their refining and chemical analysis (see Table 6). In order to obtain samples with a specific humidity value we had to find a reliable method to hydrate or dehydrate the sample. At first we tried introducing the bran sample into an acclimatised chamber that was set up to have an inner air humidity equal to the one desired in the sample. This proved to be completely inefficient, since that air humidity within the machine

was always inferior to that one in the storage room where the bran had been previously kept, causing the sample always to dehydrate.

The most convenient option proved to be the use of the humidity values known after the performance of the air oven method in the raw material pre-analyses to know the exact amount of water to be added by simply mixing it drop by drop into the sample with the help of a mixer. For some cases, the humidity in the bran was higher than that one desired for the extraction and for that reason in had to be dehydrated with the help of a dry oven. The new humidity values were then recalculated (air-oven method) and the necessary amount of water was added as described before.

The extract yields were, as expected, higher for the normal bran (between 3 and 5 g per 100 g of wheat bran generally), than for the food bran (around 2.5 g per 100 g of wheat bran), since the second type already showed to have a significantly lower amount of fat during the pre-analysis. However, wheat bran extracts coming out of the separator of a CO_2 extracting unit come out in a semi-solid form (see Figure 15) and they are not composed uniquely by oil but also contain high amounts of water, waxes and other compounds. This makes the physical separation necessary.

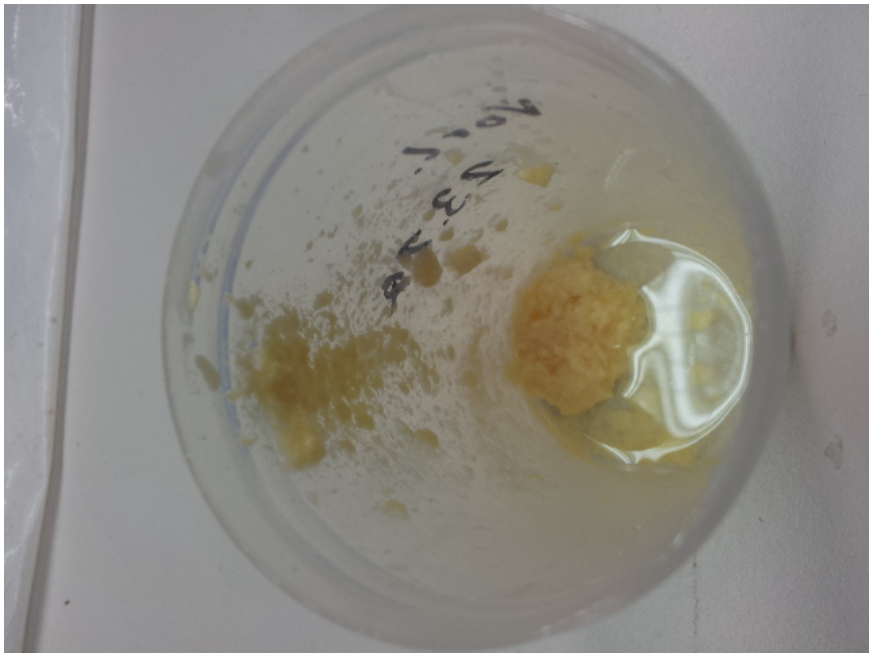


Figure 15: Wheat bran extract as it looks at its exit of the separator in the CO_2 extracting unit.

The extractions were performed with a supercritical CO_2 extraction counting with a pump and extractor and two separators, from which only one of them was used. This type of extraction revealed to be sometimes inefficient (at least for the type of raw material used in this experiment), since a big part of the extraction remained stuck to the walls and exit valve of the separator due to the low temperatures achieved at this point and the high content of the extraction in waxes and other compounds.

Measuring the weight loss in the bran-look-alike by-product also shows that indeed, there were very big extract losses (see Table 7). The weight loss was much greater than the weight of the extract collected at the exit of the separator. An average of 54.5% of the extract is lost in the process, being such losses always between 30% and 70%. A major part of this extraction losses may however not be wheat bran extract in its essence but part of the water contained in the bran that turns into vapour at certain steps of the extraction process. Even so, the losses are still very high and it raises important questions concerning the suitability of the supercritical CO_2 extraction technology to obtain extracts using wheat bran as raw material. A classical liquid solvent extraction using organic solvents such as toluene, propane or hexane, which is nowadays very broadly used in the industry, may in this case be more convenient.

Table 7: Extract yields and losses in the extractions.

Sample	*Bran type*	*Water content [%]*	*Press. [bar]*	*Temp. [ºC]*	*Weigh in [g]*	*Weigh out extract [g]*	*Yield extract [g/100 g bran]*	*Weigh out B.P. [g]*	*Weight loss [g]*	*Losses [g]*	*Losses [%]*
1	Nor.	11	450	40	500	15.4	3.1	459.7	40.3	25.0	61.7
2	Nor.	9	350	40	500	18.3	3.7	473.6	26.4	8.1	30.7
3	Nor.	11	350	50	500	18.4	3.7	454.1	46.0	27.5	59.9
4	Nor.	13	450	50	500	18.7	3.7	444.7	55.3	36.6	66.2
5	Nor.	13	350	60	500	25.4	5.1	438.6	61.5	36.1	58.7
6	Nor.	11	450	60	500	19.8	4.0	448.5	51.5	31.7	61.5
7	Nor.	9	250	50	500	16.7	3.3	471.4	28.6	11.9	41.7
8	Nor.	9	450	50	500	15.6	3.1	472.6	27.5	11.9	43.2
9	Nor.	13	250	50	500	21.6	4.3	444.2	55.8	34.3	61.4
10	Nor.	11	350	50	500	18.7	3.7	450.2	49.8	31.1	62.4
11	Nor.	9	350	60	500	21.0	4.2	453.8	46.2	25.2	54.6
12	Nor.	11	250	40	500	14.3	2.9	461.0	39.0	24.7	63.3
13	Nor.	11	250	60	500	25.0	5.0	446.0	54.1	29.1	53.8
14	Nor.	11	350	50	500	16.8	3.4	452.5	47.5	30.7	64.7
15	Nor.	13	350	40	500	15.5	3.1	452.2	47.8	32.3	67.6
16	Nor.	13	450	60	500	35.3	7.1	444.3	55.7	20.5	36.7
17	Food	13	450	60	350	8.7	2.5	312.9	37.2	28.4	76.5
18	Nor.	12.7	450	60	500	34.7	7.0	444.5	55.5	20.8	37.4
19	Food	12.7	450	60	350	8.9	2.6	312.7	37.3	28.3	76.0
20	Nor.	9	449.9	40	500	13.3	2.7	480.0	20.0	6.7	33.5
21	Food	9	449.9	40	350	2.4	0.7	346.4	3.6	1.3	34.8

4.3 Oil sample preparation

Measuring of the different phases after the centrifugation and manual separation revealed the extracts were composed mostly by oil but contained also very high amounts of water (see Figure 16).

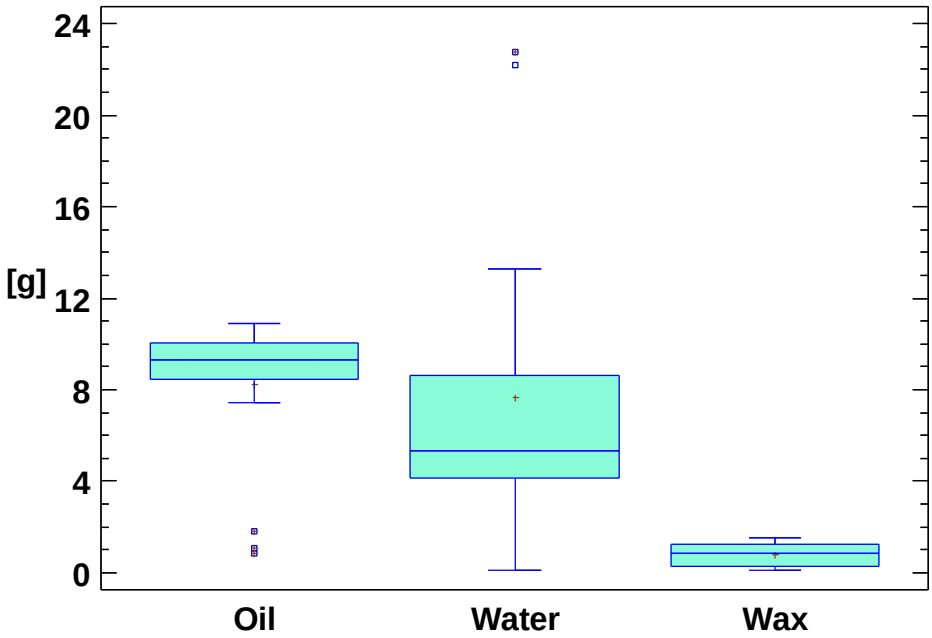


Figure 16: Yield of oil, water and wax in the normal bran.

This way, an average of 51% of the sample was oil and 35% of its content was water. The remaining 15% were waxy components. Extracts coming out of the food bran however, contained higher amounts of water (41%), superior to its oil contents (32%), whilst the solid waxy phase represented around 27% (see Table 8). However, it is difficult to determine whether the extraction yields are accurate enough since, due to the waxy nature of the extract and its very low temperature at the exit, an important part of it, especially a mixture of the waxy and oil phases remains stuck to the walls of the exit valve in the extracting unit's separator making it impossible for it to be collected. The water phase seemed to present no problems at the time of evacuating the separator and was already clearly visible at the time the sample came out of the separator since it did not get mixed with the other two phases due to their hydrophobicity. It is possible therefore, that a greater part of the waxy and oil phases remains in the separator while the water phase is more easily evacuated out of it and that could be the reason for the inconsistency of our results (see Table 8).

Table 8: Percentages of the three phases obtained during separation of the wheat bran extracts.

Sample	*Bran type*	*Water content [%]*	*Press. [bar]*	*Temp. [ºC]*	*Oil in extract [%]*	*Water in extract [%]*	*Wax in extract [%]*
1	Nor.	11	450	40	62.28	27.41	10.30
2	Nor.	9	350	40	59.54	28.76	11.70
3	Nor.	11	350	50	54.54	35.09	10.37
4	Nor.	13	450	50	45.10	39.75	15.14
5	Nor.	13	350	60	39.46	52.34	8.19
6	Nor.	11	450	60	38.093	43.59	18.31
7	Nor.	9	250	50	60.86	24.25	14.89
8	Nor.	9	450	50	61.96	25.59	12.44
9	Nor.	13	250	50	48.52	43.78	7.70
10	Nor.	11	350	50	48.96	33.46	17.58
11	Nor.	9	350	60	48.14	38.52	13.33
12	Nor.	11	250	40	59.39	21.70	18.91
13	Nor.	11	250	60	40.41	47.58	12.00
14	Nor.	11	350	50	52.06	31.34	16.60
15	Nor.	13	350	40	57.88	26.68	15.44
16	Nor.	13	450	60	26.40	64.47	9.12
17	Food	13	450	60	12.35	59.89	27.75
18	Nor.	12.7	450	60	28.85	63.84	7.31
19	Food	12.7	450	60	9.20	59.64	31.16
20	Nor.	9	449.9	40	55.80	26.44	17.75
21	Food	9	449.9	40	75.68	4.24	20.08

Oil yields were significantly different for the two types of bran used in this research as the data of the raw-material pre-analysis had already suggested. For the normal bran, around 2 g of oil were obtained out of every 100 g bran every time whilst for the food bran, the amount of oil obtained was always between 0.3 and 0.5 g of oil per 100 g bran (see Figure 17). This could be due to both brans coming from different harvests at different moments of the year (i.e. wheat cultivated during winter is supposed to contain a higher amount of water and lower amount of fat) or to a different variety of wheat.

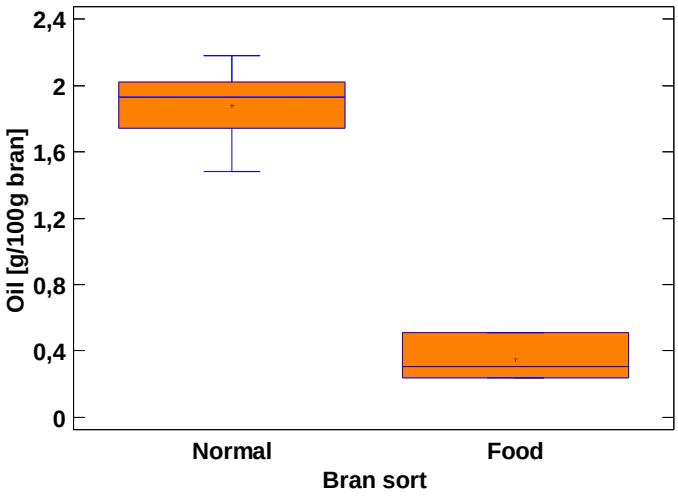


Figure 17: Comparison of oil yields between the normal and the food bran used in this research.

The results however, showed that CO_2 extraction is not as efficient as a common extraction with a conventional solvent, since the results given by the raw material pre-analysis stated that 4.3 g of oil could be extracted out of a 100 g of normal bran and 2.4 g out of the food bran using a soxhlet method. This could be due once more to the great losses experienced during extraction, as an important part of the wheat bran extract remained stuck to the walls and exit valve of the separator due to its low temperature at that point and the high contents of certain compounds such as waxes that the extract itself has. The analysis of the bran-look-alike sub-product will shed a bit more light over this matter since a soxhlet analysis of this material will reveal how much fat is left in it after the CO_2 extraction.

A statistical analysis of the oil yields revealed that pressure was the most relevant parameter concerning the oil yield (see Figure 18). It was at the lowest pressures (around 250 bar) when the higher yields of oil where obtained. Temperature was also affecting the yields in an inversely proportional way, since the highest yields where also obtained at temperatures of around 40º C, the lowest in which this experiments took place. However, the oil yields differed very little between those experiments performed at lower and those at higher temperatures and taking into consideration that there were high losses at any extraction condition, it could be considered that temperature was not a determining factor for the oil yields.

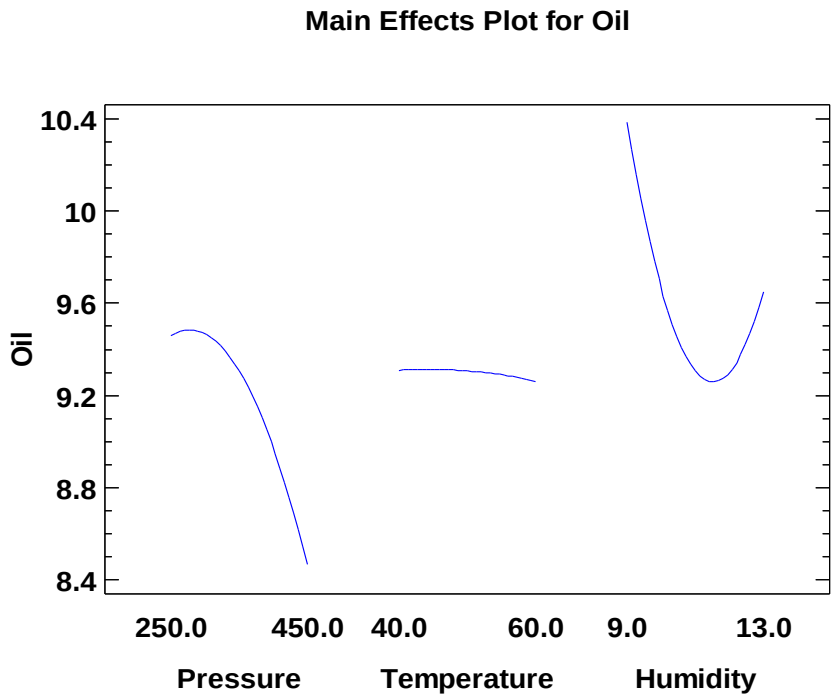


Figure 18: Oil yields at different under different extraction conditions of pressure, temperature and humidity.

Indeed, as it can be seen in Figure 19 where the oil yields at different conditions of temperature and pressure and at a constant humidity of 11%, the greatest results were obtained at conditions of low pressure (250 bar) and high temperature (60 ºC).

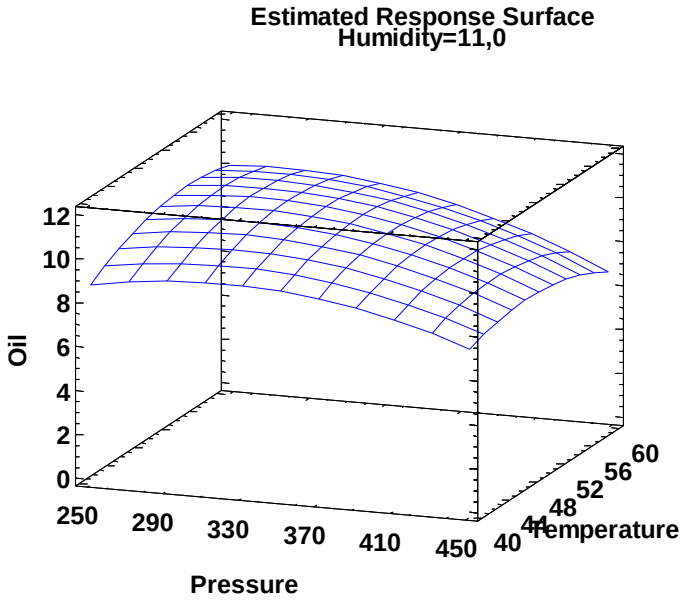


Figure 19: Three-dimensional simulation for oil yields different extraction conditions of temperature and pressure at a humidity of 11%.

According to the data, humidity had as well an impact on the wheat bran oil yields. Those were greater at conditions of low humidity/water content in the sample (9% at its lowest in our experiment) (see Figure 18). However the results where not inversely proportional to the humidity values, since the lowest yields were obtained at conditions of moderate humidity (around 11%) whilst at the highest humidity values the oil yields were moderate.

4.4 Chemical analysis of the oils

4.4.1 Acid Value

In this research, the samples extracted with supercritical CO_2 were analysed for the acid value together with a laboratory-scale extraction using petroleum ether (soxhlet) as well as commercial olive oil, in order to compare their acidity. The reason we performed this parallel olive oil analysis was that this product is very well described in the literature. Comparing our data of the olive oil with that in the literature allowed us to see if our method for the measure of the acid value was correct.

As it can be seen in Table 9 the acid value analysis revealed that CO_2 extracted wheat oil samples had very high acid values and acidity, even after a wash with sodium sulfate to eliminate their dissolved water. Since the olive oil results were within the expected values, the results suggest that the reason for such acidity is the high presence of free fatty acids as a result of hydrolysis. Wheat bran oil extracted with the soxhlet method had as well high acidity values but still way lower that in the CO_2 extracted samples, suggesting the problem may reside on a combination of the CO_2 extraction process, because of the high pressures achieved in the process, and the raw material itself.

Table 9: Acid value and acidity of wheat bran oil extracted and treated with diverse methods compared to a standard commercial olive oil.

Sample type	***Acid value [g KOH/g fat]***	***Acidity [%]***
Wheat bran oil (CO_2 extraction)	*90.3 – 91.8*	*46.0 – 48.6*
Wheat bran oil (CO_2 extraction; pre-washed)	*87.9 – 90.3*	*45.4 – 47.8*
Wheat bran oil (Soxhlet extraction)	*33*	*17*
Olive oil	*2.5*	*1*

The reason the wheat bran could be partially responsible for such hydrolysis could be the presence of enzymes such as the lipases, which would hydrolyse the triglycerides. For this reason we ran several CO_2 extractions using a special type of wheat bran as a raw material. Such bran is pre-treated with steam to de-activate enzymes.

4.4.2 Thin-Layer Chromatography

In this research, the samples extracted with supercritical CO2 were analysed for the TLC together with some laboratory-scale extractions using petroleum ether (soxhlet) and hexane as well as commercial olive oil, in order to compare their behaviour when performing a thin-layer chromatography. All samples extracted with CO_2 showed high levels of hydrolysis and very high amounts of free fatty acids as a result (see Figure 20). This is an explanation to the high acid values and acidity levels found in the previous experiment.

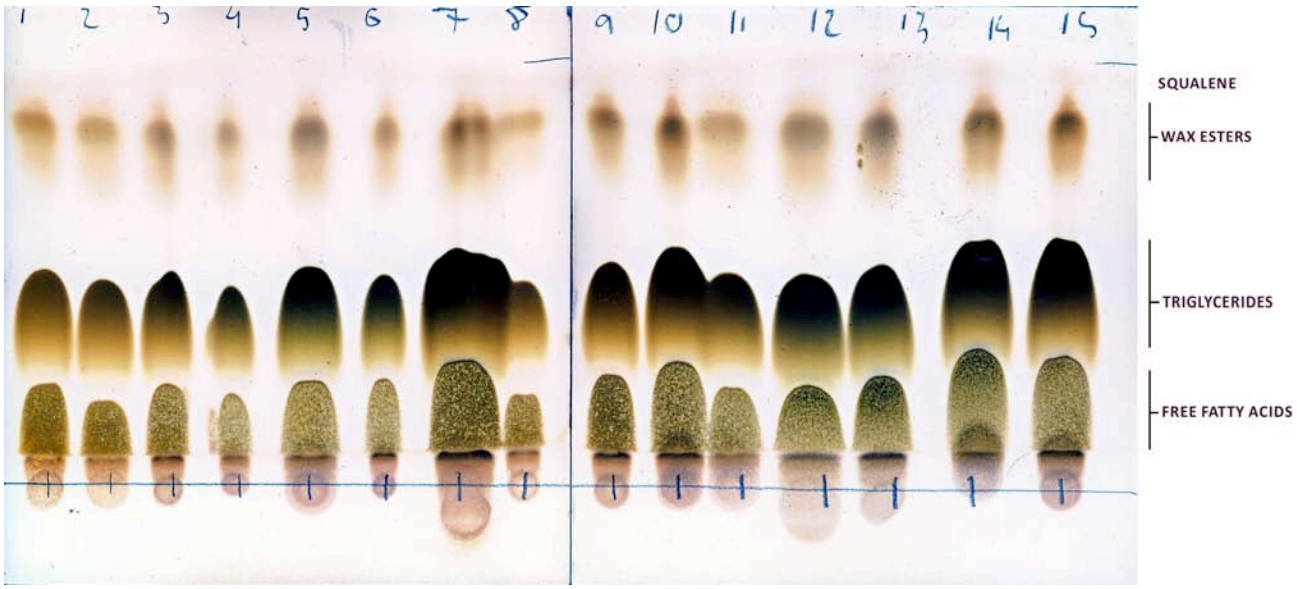


Figure 20: Thin layer chromatography for several samples.

As it can be observed in Figure 21 the commercial sample of olive oil used as a standard, did not present visible levels of hydrolysis sub-products meaning the problem was not located in the analytical method but in the sample instead. Samples extracted with the Soxhlet method (petroleum ether) showed more moderate levels of hydrolysis, which matches with the findings in the acid value experiment. Samples extracted with hexane showed similar results, with lower amounts of free fatty acids as the oil samples extracted with supercritical CO_2 but still presenting some products of hydrolysis (see Figure 22). This points out that the major cause of the hydrolysis may reside in the supercritical CO_2 extraction process, most probably due to the high pressures achieved in the process.

Although the petrol ether and hexane extracted samples had lower amounts of free fatty acids, it still presented some hydrolysis that could be due to the work of certain enzymes present in the wheat bran such as the lipases. For this reason we started conducting CO_2 extractions using a special type of wheat bran that is pre-treated with steam to inactivate such enzymes.

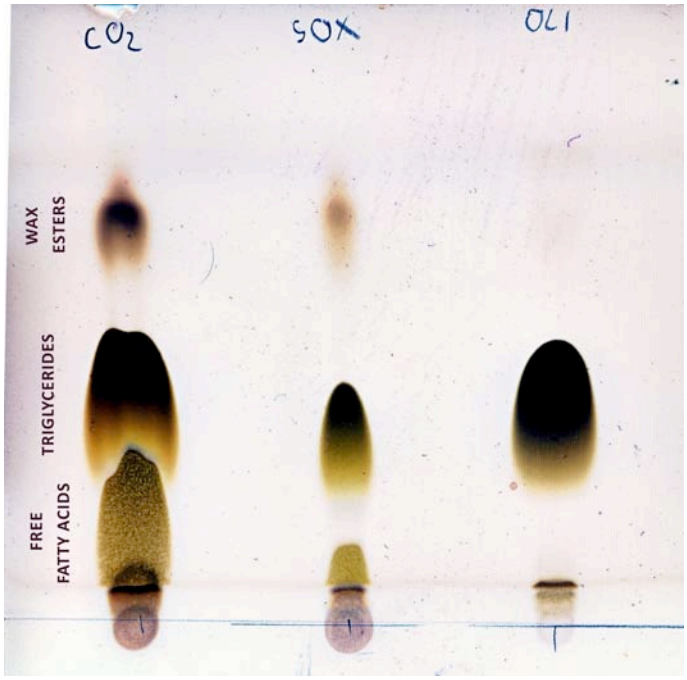


Figure 21: Thin layer chromatography comparison between a wheat bran oil sample extracted with supercritical CO_2, a sample extracted with the soxhlet (SOX) method and a commercial type of olive oil (OLI).

Results however were not very encouraging either for the special type of wheat bran called food bran, which is treated with steam. The levels amounts of free fatty acids as a result of a previous hydrolysis were still as high as in those samples coming from normal bran suggesting the cause may reside entirely in the CO_2 extraction process.

CHAO-RUI et al. (2008) established a method to use the pressure, temperature and solvent consumption parameters in the supercritical CO_2 extraction in order to reduce the acidity in the extract by minimizing content of free fatty acids. A success of up to 97.8% free fatty acid removal was obtained under conditions of 250 bar, 79.85 ºC and 2700 g of carbon dioxide usage. Further research has to be done in order to obtain similar success with wheat bran oil extracts.

The TLC analysis allowed as well the visualization of other compound groups such as triglycerides, diglycerides, sterols or wax esters (see Figure 22). The amount of triglycerides appeared to be significantly lower in wheat bran oil samples than in the commercial type of olive oil used as a standard and it was higher in those samples extracted with CO_2 than in those using a liquid solvent such as hexane. The amounts of diglycerides, and sterols were higher in wheat bran oil than in the standard olive oil. Sterols are interesting because they have the capacity to lower LDL and plasma cholesterol, because of their similar structure to these molecules. Reducing cholesterol in the organism has been demonstrated to drastically reduce morbidity and mortality. The results of the gas chromatography analysis for sterols

and stanols will shed more light over this subject. Wax esters were present in high amounts in the wheat bran oil samples, especially in those extracted using the supercritical CO_2 extracting unit, and these may be a major part of the composition of the white layer that formed as an intermediate phase between oil and water in the extractions after centrifugation (see point 3.4.1 and 4.3) and that was separated from the oil layer in order to obtain a more purified sample. Further analysis needs to be done on this part of the extract in order to obtain a more precise view of its composition.

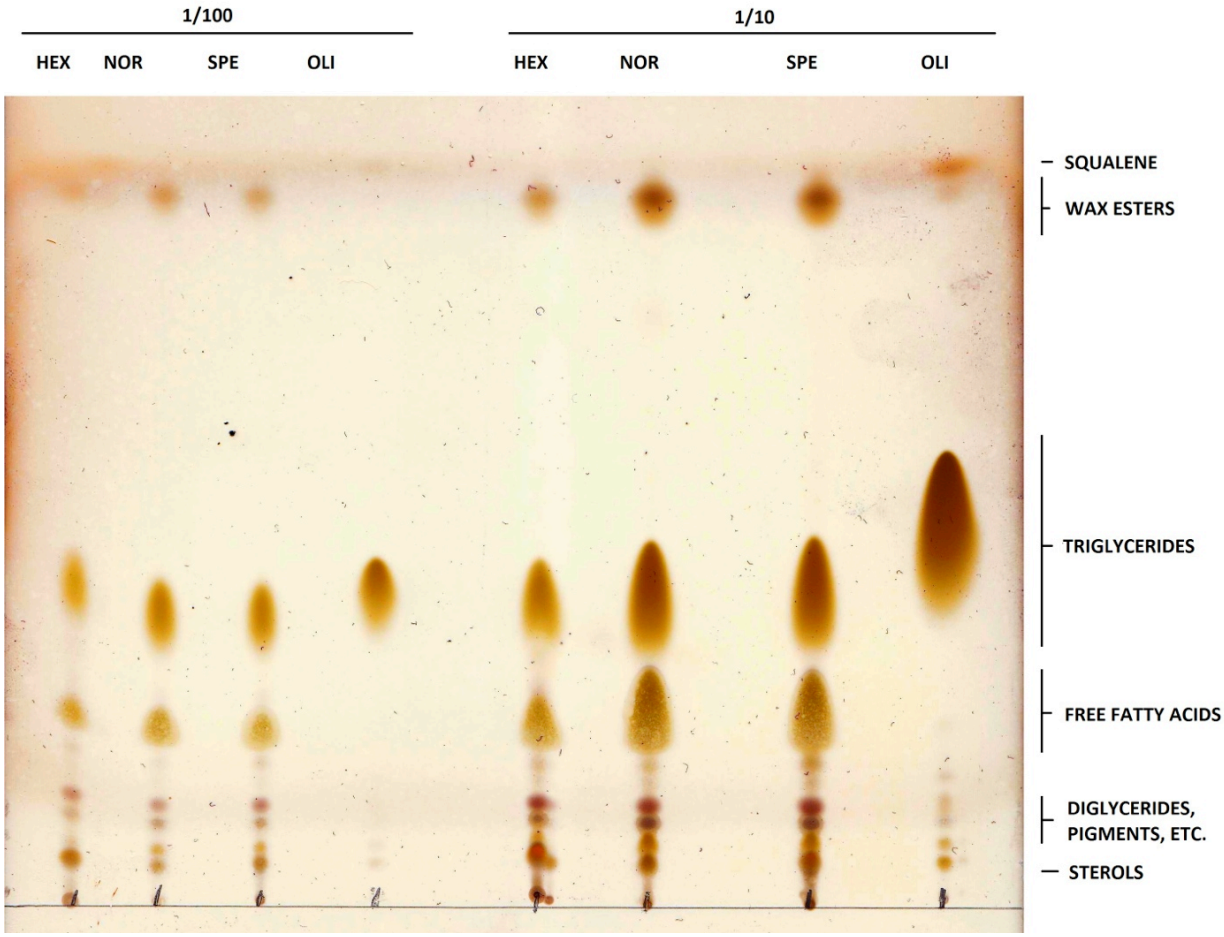


Figure 22: Thin layer chromatography comparison between oil samples extracted with supercritical CO_2 from normal (NOR) and food (SPE) brans, a sample extracted using hexane (HEX) as a solvent and a commercial type of olive oil (OLI); measured at 1/100 and 1/10 dilutions.

4.4.3 Fatty Acids Methyl Esters (FAME)

The fatty acid composition of an oil sample is interesting in the measure that it has implications in human health since oils represent up to 40% of the energy intake in western diets. Fatty acids are classified as saturated (SFA), monounsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids.

The consumption of oils rich in saturated fatty acids has been associated with cardiovascular disease by organizations such as the World Health Organization, the American Dietetic Association, the World Heart Federation, the United States Food and Drug Administration, and the European Food Safety Authority. It is recommended instead to substitute its consumption with unsaturated and mainly polyunsaturated fat, which is also believed as well to reduce the risk of other diseases such as breast cancer [PATTERSON et al., 2010]. Diets that are deficient in essential fatty acids (EFA) such as linoleic (18:2), linolenic (18:3) and arachidonic acid (20:4), are a cause for growth retardment and the appearance of dermal symptoms [CHOWDHURY et al., 2007].

Running a gas chromatography analysis of the methyl ester derivates of the fatty acids in the oil samples allowed us to determine the fatty acid profile of the CO_2 extracted wheat bran oil samples in the form of fatty acids methyl esters (FAME) (see Figure 23).

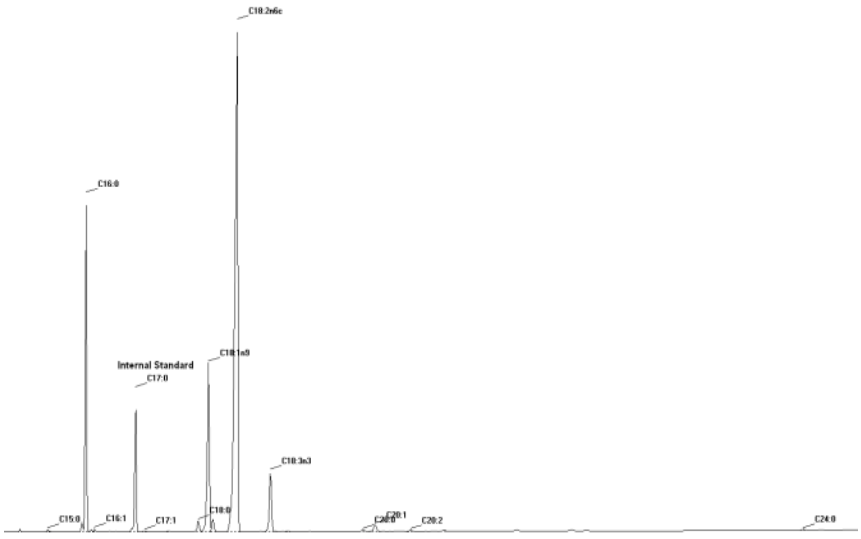


Figure 23: Gas chromatogram for the fatty acids present in a wheat bran oil sample extracted with supercritical CO_2.

According to previous studies, the major fatty acids present in wheat bran oil are in this order linoleic, palmitic, oleic and γ-linolenic. The oils contained highest percentage of linoleic acid independently of the conditions of heat and pressure in the process, in a range between 45.4 and 60%. Palmitic acid was always the second most abundant (and the most abundant within the saturated fatty acids) in amounts which ranged between 15.5 and 18.6 % (see Table 10). The fatty acid compositions were changed moderately at different extraction conditions but the results showed no significant difference between using supercritical and near-critical CO_2 extractions [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

Table 10: Major fatty acid mean values + standard deviation of wheat bran oil obtained by supercritical and near-critical CO_2 extraction [GO-WOON et al., 2010].

Pressure [bar]	*Temperature [ºC]*	*Palmitic [%]*	*Oleic [%]*	*Linoleic [%]*	*γ-linolenic [%]*
100	25	17.8±0.12	14.5±0.11	54.8±0.65	6.4±0.16
	30	17.0±0.21	14.6±0.15	54.2±0.42	7.1±0.26
	35	17.1±0.44	14.3±0.37	55.2±1.01	6.8±0.19
	40	17.9±0.32	14.2±0.26	56.4±0.63	7.1±0.29
	45	17.5±0.21	14.6±0.24	56.2±0.41	6.9±0.31
150	25	17.5±0.33	15.3±0.19	56.3±0.33	6.9±0.10
	30	17.9±0.16	15.2±0.34	55.7±0.61	6.9±0.17
	35	17.8±0.27	14.2±0.16	54.3±0.88	6.4±0.23
	40	17.2±0.37	14.6±0.26	54.4±1.23	6.2±0.21
	45	17.7±0.24	14.2±0.27	54.4±1.32	6.5±0.34
200	25	18.6±0.28	13.3±0.33	53.6±0.56	6.4±0.14
	30	17.6±0.11	14.2±0.17	52.7±0.75	6.5±0.24
	35	17.1±0.25	15.0±0.12	54.7±1.19	6.7±0.13
	40	18.3±0.26	15.9±0.39	56.8±0.89	7.0±0.29
	45	17.4±0.41	13.3±0.29	60.0±0.99	6.4±0.21
250	25	17.3±0.45	14.7±0.17	53.6±0.56	6.5±0.12
	30	17.5±0.24	15.3±0.20	55.1±1.24	6.8±0.20
	35	18.2±0.17	14.6±0.29	53.3±1.06	6.2±0.18
	40	17.3±0.42	15.2±0.38	57.1±0.95	7.2±0.32
	45	16.8±0.46	15.6±0.28	57.8±1.37	6.7±0.22
300	25	17.2±0.15	15.4±0.27	55.1±1.17	6.7±0.15
	30	17.2±0.35	14.0±0.14	54.7±0.88	7.3±0.26
	35	17.3±0.34	15.3±0.13	55.3±0.81	6.7±0.18
	40	17.3±0.49	15.3±0.34	56.1±0.72	7.2±0.07
	45	17.6±0.23	15.8±0.24	57.4±1.25	6.3±0.12

Our results however were that the variations from one sample to another were minimum, no matter what bran sort (normal or food bran) was used, or the extraction

parameters used in the extraction. In agreement with the previous studies, linoleic acid (C18:2n6c) was the most abundant of all in amounts that were always around 51%, which is within the range determined by GO-WOON et al. (2010) and KYUNG-TAE et al. (2010). It was followed by palmitic acid (C16:0), which was always around 15% a bit under the range published by the previous research groups. Oleic acid (C18:1n9) was the third most common fatty acid (around 12%) and, unlike what had been published before, γ-linolenic acid was not the fourth in amount, but α-linolenic acid (18:3n3) instead in concentrations which ranged between 4 and 5%.

As it can be seen in Table 11, samples were rich in polyunsaturated fatty acids (56%), mostly linoleic acid (C18:2n6c) as commented before, but also with a noteworthy amount of α-linolenic acid (C18:3n3) (around 4.4%). Our extractions also contained an important amount of monounsaturated fatty acids (14%), mostly oleic acid (C18:1n9) and around 16% of saturated fat, with palmitic acid (C16:0) as the main fatty acid.

Table 11: Fatty acid spectre in the CO_2 extracted wheat bran oil samples.

SATTURATED FATTY ACIDS		
C16:0 – Palmitic acid	15.0%	*16%*
C18:0 – Stearic acid	0.8%	
MONOUNSATURATED FATTY ACIDS		
C16:1n9 – Hexadecenoic acid	<0.1%	14%
C16:1n7 – Palmitoleic acid	<0.1%	
C18:1n9 – Oleic acid	13%	
C18:1n7 – Vaccenic acid	0.8%	
C20:1 – Eicosenoic acid	0.6%	
POLYUNSATURATED FATTY ACIDS		
C18:2n6c – Linoleic acid	51.3%	56%
C18:3n3 – α-Linolenic acid	4.4%	
TOTAL		86%

Organizations such as the World Health Organization, the American Dietetic Association, the World Heart Federation, the United States Food and Drug Administration, and the European Food Safety Authority recommend to substitute the consumption of

saturated fat, which associated with cardiovascular disease, with unsaturated and mainly polyunsaturated fat, which is also believed as well to reduce the risk of other diseases such as breast cancer [PATTERSON et al., 2010]. Diets that are deficient in essential fatty acids (EFA) such as linoleic and linolenic are as well a cause for growth retardment and the appearance of dermal symptoms [CHOWDHURY et al., 2007]. In this context, wheat bran oil extractions seem to be a good substitute for certain oils of vegetable origin, which are rich in saturated fatty acids and are often used in the food industry, such as palm oil (49% SFA) or coconut oil (91% SFA). The question is whether a method could be designed to obtain wheat bran oil in an economic way and in amounts capable to supply the industry.

The fact that the total amount of fatty acids was around 86% (see Table 11) is due to the presence of other components, majorly products of hydrolysis such as free fatty acids, impurities that were not separated during the refining process such as wax esters, as it was noted in the previous thin-layer chromatography experiment, and traces of water. This impurities in our samples is the reason why the amount of some fatty acids was a slightly under the ranges published by GO-WOON et al. (2010) and KYUNG-TAE et al. (2010) when calculating the percentage. If only the fatty acid methyl esters are considered, the results agree with each other in this term.

Modifying the extraction conditions in the process (temperature, pressure, water content of the sample) did not have a relevant impact in the fatty acid profile of the samples. Extraction conditions of low humidity and high temperature seemed to reduce the amount of saturated fat in the sample, although that was achieved in a very limited way (see Figure 24). However, levels of polyunsaturated fat also seemed to decrease under conditions of high temperature and low humidity, together with high pressure (see Figure 25), which limits the positive effect of reducing the concentration of SFA in the first place.

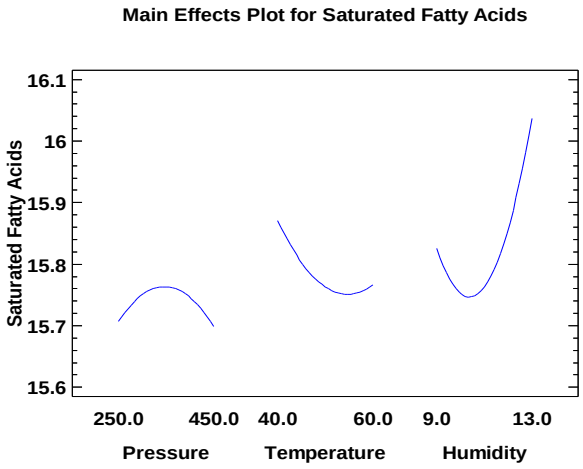


Figure 24: Variations in the amounts of saturated fatty acids at different conditions of extraction.

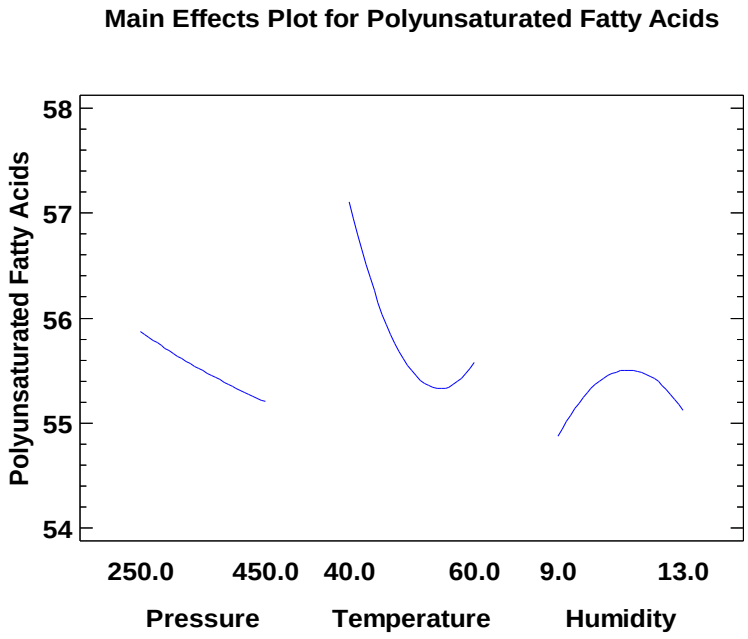


Figure 25: Variations in the amounts of polyunsaturated fatty acids at different conditions of extraction.

4.4.4 Sterols (Gas Chromatography)

Phytosterols, phytostanols and their esters are a group of steroid alcohols and esters that naturally occur in plants. The B-ring of the steroidal moiety is unsaturated in the 5-6 position in phytosterols and saturated in phytostanols. Stanols, however, occur in lower amounts in the diet. Some phytosterols and phytostanols may be extracted as esters of fatty acids. The fatty acid ester chain may be saturated, mono- or polyunsaturated depending on the source of the plant oil. Most commonly consumed phytosterols are sitosterol, stigmasterol and campesterol, which are found in important amounts in vegetable oils in the form of steryl esters. Less important sources of sterols are cereals, nuts and vegetables [PIIRONEN et al., 2000].

The nutritional interest in phytosterols locates in the fact that they are essential in the composition of all eukaryotic cells and therefore of many living organisms including humans. Their labour in the membrane is to control its fluidity and permeability and some of them act as signal transductors. Another important nutritional interest of sterols derives from the fact that the sterols have the capacity to lower LDL and plasma cholesterol, because of their similar structure to the aforementioned molecules. Phytostanols have the same plasma cholesterol lowering abilities and, unlike sterols, do not cause an increase in plasma levels, which can be detected in plasma. Reducing cholesterol in the organism has been demonstrated to drastically reduce morbidity and mortality and therefore, phytosterols and

phytostanols have an interest since they have the ability to act as natural preventive dietary products [PIIRONEN et al., 2000].

Our samples were found to contain important amounts of plant sterols and stanols in amounts that were hanging about the 2 g every 100 g of oil sample (see Figure 26). β-sitosterol was by far the most abundant one in amounts that were close to 1 g every 100 g of oil. It was followed by stigmasterol (around 350 mg/100 g of oil), stigmastanol (around 250 mg/100 g of oil) and sitostanol (over 200 mg/100 g of oil) (see Table 12). Therefore, we could say that wheat bran oil could have an interesting use as a nutritional source for phytosterols and phytostanols and have a positive impact on human health.

Unfortunately though, it was impossible to perform sterol analysis of the special food bran during this research, due to the very limited amount of sample that was obtained from the extractions with this type of bran. It is to be expected though, that the sterol contents in the oil extractions of this bran should not differ very much from those in the normal bran, since the only difference between them is a special steam treatment to get rid of certain enzymes.

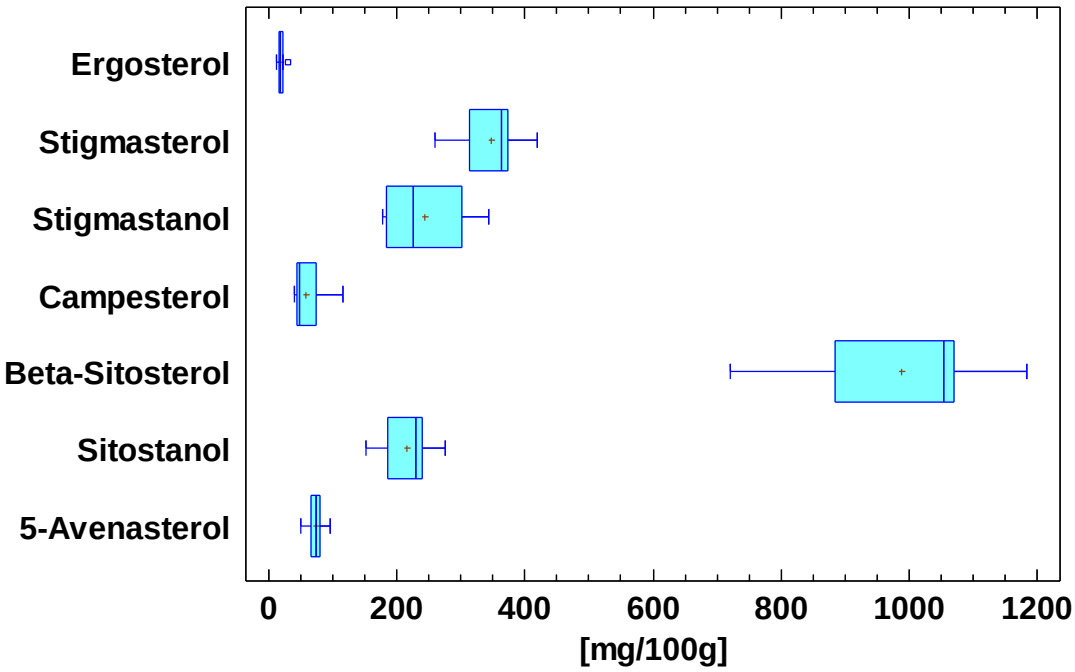


Figure 26: Amount of sterols present in the wheat bran oil samples.

Table 12: Amounts of sterols and stanols present in the wheat bran oil samples.

Sample	*Water content [%]*	*Press. [bar]*	*Temp. [ºC]*	*Ergosterol [mg / 100 g]*	*Stigmasterol [mg / 100 g]*	*Stigmastanol [mg / 100 g]*	*Campesterol [mg / 100 g]*	*β-Sitosterol [mg / 100 g]*	*Sitostanol [mg / 100 g]*	*5-Avenasterol [mg / 100 g]*	*Total sterols [mg / 100 g]*
1	11	450	40	18.9	289.8	178.6	88.6	817.0	187.0	63.6	1643.5
2	9	350	40	22.5	373.5	233.7	44.6	1052.8	229.2	75.4	2031.7
3	11	350	50	14.8	314.7	226.7	83.3	883.8	187.2	67.4	1777.9
4	13	450	50	19.8	419.0	301.0	50.0	1183.1	275.4	96.1	2344.4
5	13	350	60	21.5	391.2	306.4	59.3	1092.3	252.9	82.7	2206.3
6	11	450	60	16.6	337.6	343.8	45.9	923.6	197.3	72.1	1937.0
7	9	250	50	11.6	260.9	183.4	116.4	721.4	152.0	51.1	1497.0
8	9	450	50	22.1	296.5	184.9	75.3	811.2	171.2	58.9	1620.2
9	13	250	50	17.1	343.4	275.9	40.4	948.8	206.3	72.4	1904.2
10	11	350	50	22.1	372.6	296.4	44.8	1028.5	232.8	75.0	2072.1
11	9	350	60	29.8	401.0	334.9	50.0	1068.9	239.8	79.6	2204.1
12	11	250	40	18.4	313.6	180.9	45.9	1066.1	180.9	68.1	1873.9
13	11	250	60	18.2	371.7	207.1	48.4	1117.4	255.6	83.2	2101.4
14	11	350	50	17.3	364.2	207.4	44.4	1055.4	239.5	78.2	2006.4
15	13	350	40	19.7	364.5	196.1	46.7	1058.8	238.0	79.0	2002.9
			Average	19	347	244	59	988	216	74	1948

Some studies made in the past stated that it is possible to extract oils with high content in tocopherols from wheat bran using supercritical (<31.1°C) and near-critical (<31.1°C) CO_2 extractions, giving the final product an added value. The amount of these antioxidant in the oil extracted is dependent on the extraction conditions. α- and β-tocopherol contents (measured with HPLC) and the total phenolic content (TPC) in general, increased both with increasing temperature and pressure. Increasing the pressure increases de density, which allows the solvating CO_2 to dissolve more solutes. Increasing the temperature increases the diffusivity and decreases the viscosity, which makes the solute more soluble [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

The statistical analysis of our data reveals that variations in the parameters during the CO_2 extraction did have an impact on the phytosterol/phytostanol contents of the samples. In this manner, the highest yields were obtained in those samples extracted at the highest temperatures used in our experiments (60 ºC) as well as the highest water contents (13%). Additionally, pressure seemed to have its highest impact at moderate values (around 350 bar) (see Figure 27).

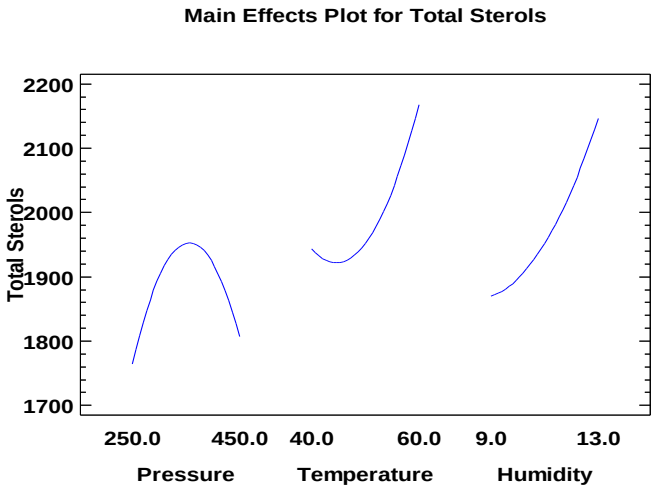


Figure 27: Total sterol yields under different extraction conditions of pressure, temperature and humidity.

In Figure 28 can be seen as well that best results are obtained at conditions of moderate pressure and high temperature in a simulation where the water content in the sample is considered to be always 11%.

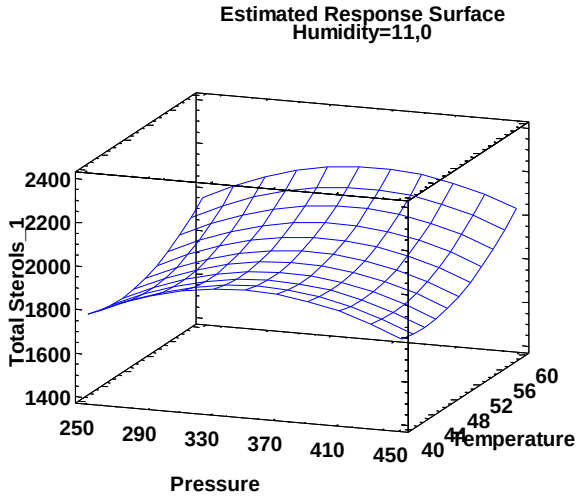
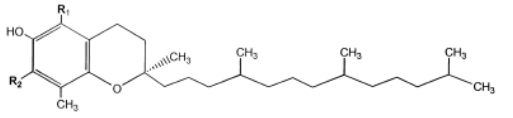


Figure 28: Three-dimensional simulation for total sterol yields at different extraction conditions of temperature and pressure at a humidity of 11%.

4.4.5 Tocols (High Pressure Liquid Chromatography)

Tocols or the vitamin E group of compounds comprises four tocopherols, and four tocotrienols, all of which have significant antioxidant properties. These lipophilic vitamins can be found in most non-processed seed oils and, when present in high concentrations, significantly increase the value of the oil [GUNSTONE, F.D et al., 2007].

Tocols are fat-soluble vitamins and antioxidants of phenolic origin. The difference between tocopherols and tocotrienols is in their side chain. Tocopherols have saturated side chains, while those in tocotrienols contain double bonds and they are therefore unsaturated (see Figure 29). Unlike tocopherols, which are also found in nuts and vegetable oils, tocotrienols are mostly concentrated in cereal grains and their oils and in palm oil as well. Both tocopherols and tocotrienols are classified with the Greek letters α, β, δ and γ, which indicate the number and position of the methyl substitution on the chromanol ring (see Figure 29). Tocols are well known for their antioxidative effect, and because of this reason they are believed to reduce cardiovascular disease and cancer by scavenging free radicals. The physiological activities of tocotrienols suggest them to be superior to those in α-tocopherol in many situations. α- tocotrienol has been observed in vitro to have a remarkably higher antioxidant activity against lipid peroxidation than α-tocopherol. Moreover, tocotrienols have been reported to help cancer prevention and to reduce plasma cholesterol levels as well as other lipid and non-lipid related risk factors for cardiovascular disease. This is a relevant discovery, as the formulation of most of the existing Vitamin E supplements may be questionable, being those composed mostly of α-tocopherol [THERIAUT et al., 1999; SARMENTO et al., 2006; KAMAL-ELDIN et al., 2009].



	R_1	R_2
α-Tocotrienol/ Tocopherol	CH_3	CH_3
β- Tocotrienol/Tocopherol	CH_3	H
γ- Tocotrienol/Tocopherol	H	CH_3
δ– Tocotrienol/Tocopherol	H	H

Figure 29: Structures of various homologs of tocopherol and tocotrienol [THERIAUT et al., 1999].

Some studies made in the past stated that it is possible to extract oils with high content in tocopherols from wheat bran using supercritical (<31.1°C) and near-critical (<31.1°C) CO_2 extractions, giving the final product an added value. The amount of these antioxidant in the oil extracted is dependent on the extraction conditions. α- and β-tocopherol contents (measured with HPLC) and the total phenolic content (TPC) in general, increased both with increasing temperature and pressure (see Table 13). Increasing the

pressure increases de density, which allows the solvating CO_2 to dissolve more solutes. Increasing the temperature increases the diffusivity and decreases the viscosity, which makes the solute more soluble [GO-WOON et al., 2010; KYUNG-TAE et al., 2010].

Table 13: Total phenolic content (TPC) and α- and β-tocopherol contents of wheat bran oil at different extraction conditions [KYUNG-TAE et al., 2010].

Temperature [ºC]	*Pressure [bar]*	*Tocopherols [mg/100 g oil]*		*TPC [mg/100 g oil]*
		α- Tocopherol	**β-Tocopherol**	
40	100	37.7	25	123
	150	52	31	146
	200	61	35	181
	250	77	42	206
	300	97	62	239
50	100	51	34	108
	150	87	41	166
	200	100	59	198
	250	129	62	236
	300	114	65	289
60	100	71	37	98
	150	94	42	187
	200	114	63	229
	250	122	69	292
	300	148	74	357

Comparing both sorts of wheat bran used in this research brings out that the special food bran had slightly higher tocol contents than the normal bran (see Figure 30). The difference between the two raw materials locates in a special treatment with steam that the food bran receives to de-activate certain enzymes, whilst the supplier and wheat sort and origin are supposed to be the same. Therefore, these minor differences are more likely to be a result of both brans coming from different harvests of wheat from different times of the year than because of the pre-treatment itself.

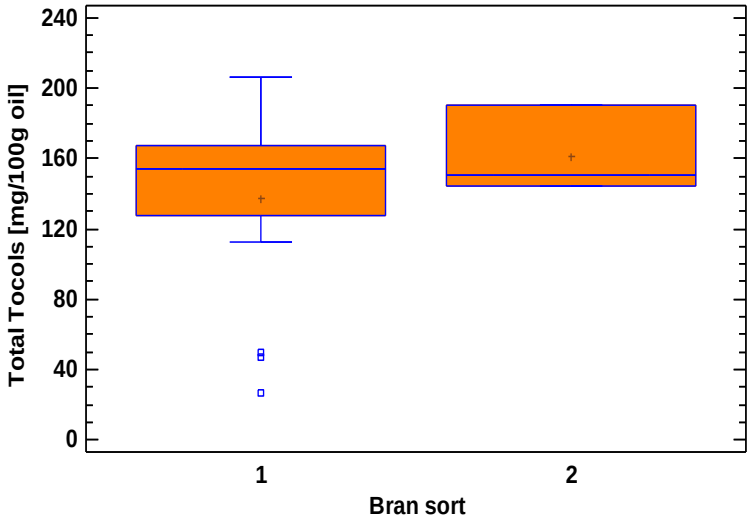


Figure 30: Comparison of total tocol contents between the normal wheat bran (1) and the food wheat bran (2) used in this research.

Around 150 mg of tocols could be found every 100 g of oil sample. Among all the tocols identified, β-tocotrienol was by far the most common with around 90 mg per 100 g of oil (see Figure 31). It was followed by α and β-tocopherol, which were both found in amounts close to the 25 mg every 100 g of sample. The last tocol detected in our analysis was α-tocotrienol, and this was present in amounts around the 15 mg per 100 g of oil (see Table 14).

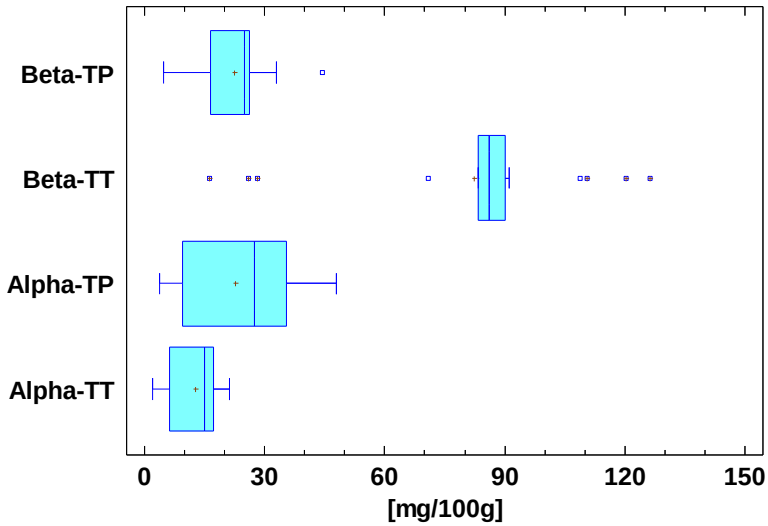


Figure 31: Amount of tocols present in wheat bran oil samples.

Table 14: Amounts of tocols present in the wheat bran oil samples.

Sample	*Bran type*	*Water content [%]*	*Press. [bar]*	*Temp. [ºC]*	*α-Tocopherol [mg / 100 g]*	*α-Tocotrienol [mg / 100 g]*	*β-Tocopherol [mg / 100 g]*	*β-Tocotrienol [mg / 100 g]*	*Total tocols [mg / 100 g]*
1	Nor.	11	450	40	29.6	15.6	25.7	84.5	155.4
2	Nor.	9	350	40	31.1	16.7	26.7	87.6	162.1
3	Nor.	11	350	50	27.6	15.1	25.0	84.8	152.6
4	Nor.	13	450	50	35.6	19.0	25.0	83.4	163.0
5	Nor.	13	350	60	39.1	20.8	24.1	86.3	170.2
6	Nor.	11	450	60	38.6	20.1	25.6	87.0	171.3
7	Nor.	9	250	50	38.6	19.8	26.1	84.9	169.4
8	Nor.	9	450	50	32.3	17.2	28.0	89.4	167.0
9	Nor.	13	250	50	48.0	16.8	25.5	70.8	161.0
10	Nor.	11	350	50	27.6	13.9	25.4	86.1	153.1
11	Nor.	9	350	60	41.7	21.4	32.9	110.4	206.5
12	Nor.	11	250	40	13.5	7.7	27.8	90.1	139.1
13	Nor.	11	250	60	10.6	5.6	7.6	26.0	49.9
14	Nor.	11	350	50	3.7	2.1	4.9	16.3	27.0
15	Nor.	13	350	40	6.7	3.5	8.5	28.3	47.0
16	Nor.	13	450	60	16.8	13.5	16.6	85.9	132.7
17	Food	13	450	60	9.7	15.1	16.5	108.8	150.1
18	Nor.	12.7	450	60	5.6	5.6	18.3	83.3	112.8
19	Food	12.7	450	60	4.5	5.7	13.6	120.2	144.0
20	Nor.	9	449.9	40	6.3	6.3	23.6	91.1	127.2
21	Food	9	449.9	40	13.0	6.7	44.4	126.1	190.2
				Average	26	14	25	92	157

In the researches done by GO-WOON et al. (2010) and KYUNG-TAE et al. (2010), the amount of tocopherol in the oil extracted was variable depending on the extraction conditions. In this manner, extraction conditions similar to those used in our research the amounts were between 77 and 148 mg every 100 g of oil for the α-tocopherol and between 42 and 74 mg per 100 g of oil for the β-tocopherol (see table 13). These amounts are considerably higher to those found in our research (see Table 14). This can be seen as well by taking a look at Table 15, which compares the findings made by KYUNG-TAE et al. (2010) with ours. The reason for the lower tocol yields in our samples should not be in the extraction method, since both researches used supercritical CO_2 extracting units to obtain the oil, but it could be due to the use of a different variety of wheat bran.

Table 15: Comparison of the tocol yields at extraction pressures of 250 bar with the results published by KYUNG-TAE et al. (2010).

Temperature [ºC]	*Tocopherols [mg/100 g oil]*				*TPC [mg/100 g oil]*	*TPC [mg/100 g oil] [KYUNG-TAE et al., 2010]*
	α- Tocopherol	**α- Tocopherol [KYUNG-TAE et al., 2010]**	**β-Tocopherol**	**β-Tocopherol [KYUNG-TAE et al., 2010]**		
40	13.5	77	27.8	42	139.1	206
50	38.6 - 48.0	129	25.5 - 26.1	62	161.0 - 169.4	236
60	10.6	122	7.6	69	49.9	292

According to GO-WOON et al. (2010) and KYUNG-TAE et al. (2010), the amount of tocopherols in the oil extracted is dependent on the extraction conditions. α- and β-tocopherol contents (measured with HPLC) and the total phenolic content (TPC) in general, increased both with increasing temperature and pressure (see Table 13). Increasing the temperature increases the diffusivity and decreases the viscosity, which makes the solute more soluble. Increasing the pressure increases de density, which allows the solvating CO_2 to dissolve more solutes, however, the pressure achieved in that research were 300 bars at their highest whilst in our research, we extracted with pressures that reached the 450 bars.

Our statistical analysis of the data reveals that variations in the parameters during the CO_2 extraction did indeed have an impact on the tocol contents of the samples. Temperature, just as at the researches by GO-WOON et al. (2010) and KYUNG-TAE et al. (2010) had a positive impact in the tocol contents in the way that those samples containing the highest amount were those extracted at the highest temperatures used in this research (60 ºC) (see Figure 32). Pressure seemed to have a positive effect in the tocol yields within the oil samples at first, agreeing with the aforementioned studies, but over approximate pressures to 400 bars, this parameter had a negative effect. The water content in the sample did not seem to have a significant effect in the tocol contents of the wheat bran oil samples extracted with supercritical CO_2. Taking a look at Figure 33, regarding only variations of temperature and pressure and considering the water content/humidity in the sample as 11% best result were obtained with a combination of a temperature of 60 ºC and a pressure of 450 bars.

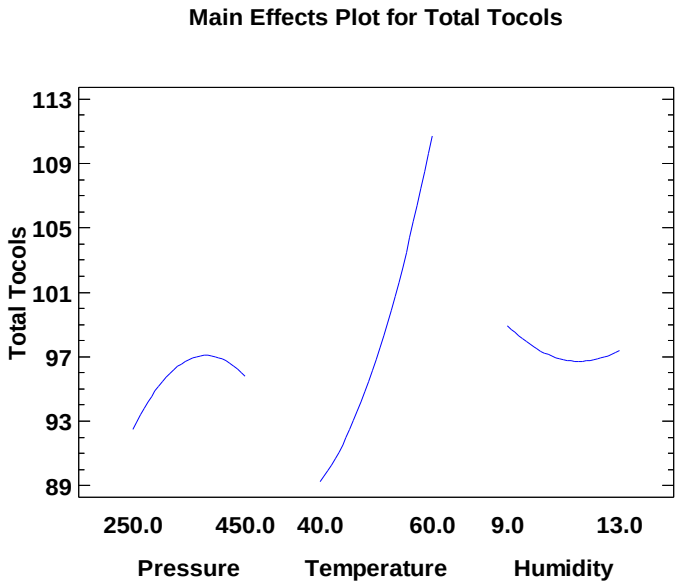


Figure 32: Total sterol yields under different extraction conditions of pressure, temperature and humidity.

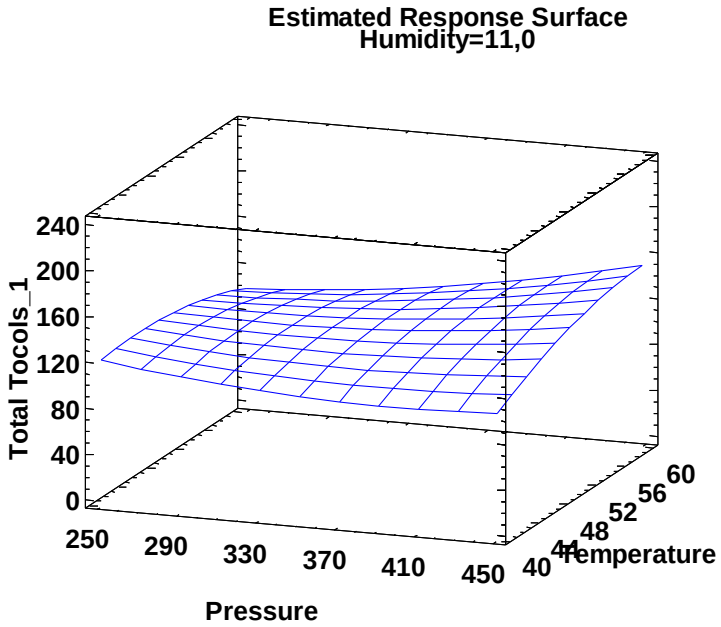


Figure 33: Three-dimensional simulation for total tocols yields at different extraction conditions of temperature and pressure at a humidity of 11%.

4.5 By-product analysis

After the CO_2 extraction, a by-product was obtained from the inside of the extractor, which was the bran used for the extraction, now defatted and slightly dehydrated. The by-product had a very similar look and feel in the hand to the one of the unprocessed wheat bran but the colour was slightly brighter. Apart from the tone, it differed nothing from it (see Figure 34).

Figure 34: Bran-look-alike by-product of the supercritical CO_2 extraction.

4.5.1 Protein – Kjedahl Method

An analysis of the by-products of every extraction revealed that they had considerably high contents of protein (around 17%) (see Table 16), making it a very interesting source of vegetable protein. The contents were very similar to those in the original bran, and this is expected, since most proteins do not dissolve in the carbon dioxide during the extraction and therefore remain in the bran. The same is expected for the dietary fibre although it was not analysed in this research. The high contents in protein and the expected high amounts of dietary fibre could give the extraction by-product a high interest in food fortification. One of the great advantages of using supercritical CO_2 as a solvent is that this evaporates after the extraction and therefore leaves no rests in the extracts or by-products, unlike organic solvents, which leave traces of toxic compounds. This advantage of CO_2 versus other solvents together with the nutritional properties of the by-product could help this last one finding a way for being sold as a high-value low-fat and protein-rich feed for farm animals.

Table 16: Average contents for dry matter, water, protein and fat in the bran-looking by-product.

	%
Dry matter [%]	95.7
Water [%]	4.3
Protein [%]	17.6
Fat [%]	0.6

4.5.2 Dry Substance – Air Oven Method

Water represented around 4% of the content in the by-product and it was found in significantly lower amounts than in the bran before the extraction. Indeed, as stated previously, the extract obtained at the exit of the separator contained high (and variable) amounts of water.

Taking a look at Figure 35, it can be observed that the temperatures achieved during the extraction had an inversely proportional effect in the amounts of water in the by-product, stating that a higher temperature had a logical dehydrating effect in the bran. In the same graphic it can be seen that, logically, the final water contents in the by-product were higher, the higher were those contents in the initial bran sample.

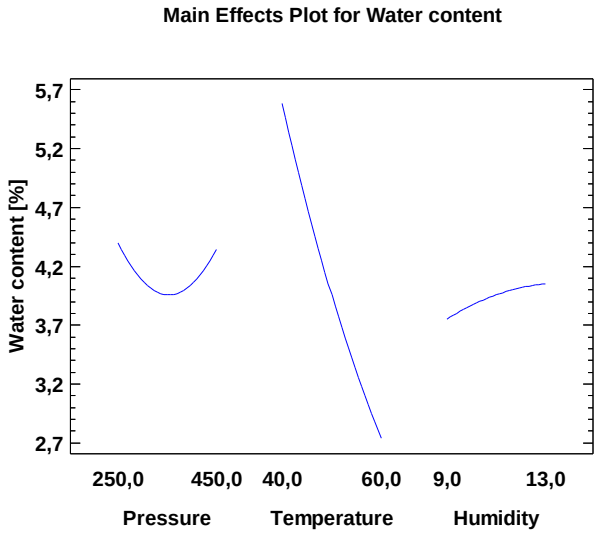


Figure 35: Water content in the bran-look-alike by-product at different extraction conditions.

4.5.3 Fat – Soxhlet Method

All three modifiable extraction parameters studied in this research (pressure, temperature and water content of the bran) had a positive effect in the defatting of the bran the higher they were according to the by-product analysis (see Figure 36). This contradicts what was found in the analysis of the extraction data (see Figure 18), where all three parameters, especially pressure and temperature had a negative effect in the oil yields the higher they were. Indeed, as it has been stated before, the extraction data was unfortunately not entirely reliable due to part of the extracts remaining stuck to the walls at the exit of the separator making it impossible for them to be collected.

A three-dimensional simulation at a constant humidity of 11% shows that better results for the defatting of the bran were achieved when using a combination of high temperature and high pressure, achieving the best results when those parameters where at their highest (60 ºC and 450 bar) (see Figure 37).

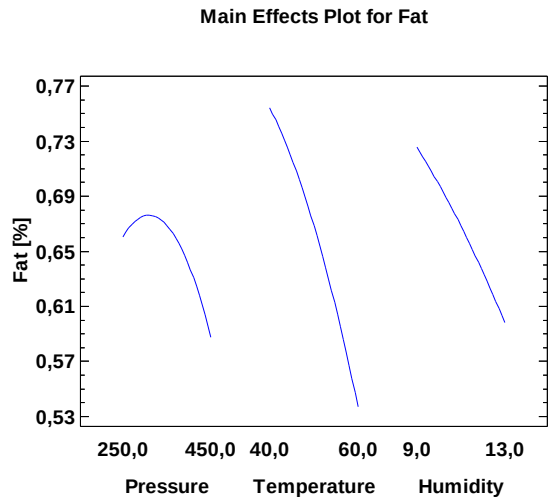


Figure 36: Fat content in the bran-look-alike by-product at different extraction conditions.

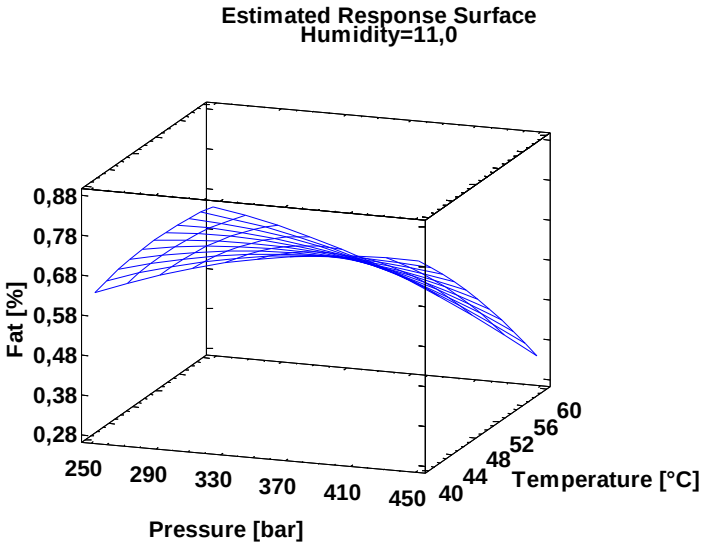


Figure 37: Three-dimensional simulation for the fat content in the bran-looking by-product at different extraction conditions of temperature and pressure at a humidity of 11%.

5 Possible future ways of study for supercritical CO_2 extractions of wheat bran oil

Some researches done in the past were focused on analyzing the levels of certain phenolic antioxidants such as tocopherols [KYUNG-TAE et al., 2010]. In our research we studied tocotrienols together with them, as well as sterols. However, there are other interesting phenolic origin antioxidants present in large amounts in wheat bran, which may end up in its oil extractions such as Alkylresorcinols [KAMAL-ELDIN et al., 2009; KORYCINSKA et al., 2009]. It may be interesting to focus a research on the levels of such natural antioxidants in the oil extracts of wheat bran.

5.1 Alkylresorcinols and Alkenylresorcinols

Alkylresorcinols are phenolic compounds possessing two hydroxyl groups at positions 1 and 3 of the benzene ring and a long alkyl chain at position 5 conformed by 15 to 25 carbon atoms (see Figure 38). The classification of the different alkylresorcinols according to the length of their side-chain can be seen on Table 17. They are mostly found in members of the *Gramineae* family, such as wheat, rye, triticale, and barley, which are the main sources for human intake, and they are mainly concentrated in the bran milling fraction of these cereal grains. The importance of Alkylresorcinols are believed to have several effects of interest on human health, such as anticancer, antimicrobial, antiparasitic, antitumor, antioxidant effects and antileukemic properties [DA CRUZ FRANCISCO et al., 2005; KAMAL-ELDIN et al., 2009; ZHU et al., 2011]. KORYCINSKA et al. (2009) stated that alkylresorcinols (C15:0 – C25:0) had a DPPH radical scavenging activity from 10 to 60% at concentrations which varied from 5 to 300 µM, and this result was not dependant on the length of the alkyl side chain.

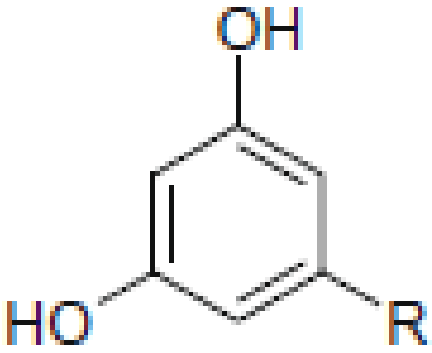


Figure 38: Chemical structure of the alkylresorcinols, being R the alkyl side chain (see table 17) of the alkylresorcinols [KORYCINSKA et al., 2009].

Table 17: Different alkylresorcinols classified by the length of their alkyl side chain.

Number of carbons in the R alkyl side chain	*Name*
15	5-n-pentadecylresorcinol (C15:0)
17	5-n-heptadecylresorcinol (C17:0)
19	5-n-nonadecylresorcinol (C19:0)
21	5-n-heneicosylresorcinol (21:0)
23	5-n-tricosylresorcinol (C23:0)
25	5-npentacosylresorcinol (C25:0)

Alkenylresorcinols are similar compounds to alkylresorcinols characterized by possessing double bonds in their alkyl side chain. According to the study made by ZHU et al. (2011), the shorter the alkyl side chain is the stronger will be the cancer inhibitory activity of the molecule. The existence of a double bond will equally strengthen this effect, making alkenylresorcinols even more interesting for this reason. It may be interesting to characterize the alkyresorcinol and alkylenresorcinol fraction in wheat bran oils.

In the extractions we performed, the main alkylresorcinol source is believed to take place in the waxy part that was mechanically separated from the oil and stored at -15 ºC. Further research could be done using that part of the extract to determine if alkylresorcinols occur in sufficient amount to find a commercial application.

5.2 Carotenoids

Carotenoids are the most widespread pigments in nature and occur in all sort of living organisms as plants, animals, bacteria and fungi. This group of molecules comprises the carotenes, a class of hydrocarbons, and the xantophyills, which are an oxygenated derivative of the first. The most important sources of carotenoids in human nutrition are fruits and vegetables, where they are found in relatively high concentrations. The most important for the human metabolism are lycopene, α- and β-carotene, lutein, zeaxanthin and β-cryptoxanthin. Their main metabolic roles are as precursors of vitamin A, and as antioxidants [ISHDA and CHAPMAN, 2009; GO-WOON et al., 2010]. GO-WOON et al. (2010) measured the

total amount of carotenoids present in a wheat bran oil extract by using the Total carotenoid essay. However, this test is not very accurate and does not characterize which mix and proportion of each carotenoid type is present in the sample. It may be interesting to characterize the different carotenoids present in a wheat bran oil sample with actual methods of HPLC.

6 Final conclusions

Supercritical extraction using carbon dioxide in gas form as a solvent is an innovative type of extraction that has been successfully used in many occasions to extract oils, flavours and other compounds out of a wide range of raw materials. One of the most remarkable advantages of this type of extraction is that, unlike other organic solvents used commonly in the industry, carbon dioxide is a non-toxic substance that leaves no traces in the extract or the by-product, since it gets evaporated soon after the extraction is finished. Its main disadvantages nowadays are the efficiency and price of the equipment, as well as the impossibility to perform extractions in a continuous manner (it requires a start and a turn off phase before and after every extraction), which hinders its possibilities to find an application in the industry.

Although CO_2 extraction offers great advantages, it did not offer the best results in our research, using wheat bran as a raw material. Although we managed to obtain a semi-refined fraction of the oil, the oil yields were too inconsistent to allow a detailed analysis of them, due to some fractions of the extract remaining stuck to the walls of the separator making it impossible for them to be collected. This made it difficult for our team to determine the most convenient extraction parameters to obtain higher yields of extract and oil. Data analysis of the extraction and of the oil remaining in the bran-look-alike by-product showed contradictory results. According to the extraction yields, the greatest results were obtained at conditions of low pressure (250 bar) and high temperature (60 ºC) whilst the analysis of the data obtained from the by-product shows that this was most successfully defatted (and thereby the oil was obtained in the extract) at extraction conditions of 60 ºC and 450 bar, as well as a high moisture content in the initial bran mix. Moreover, wheat bran extracts were not pure oil but an inhomogeneous mixture of substances. For this reason it is necessary to find a suitable raffination method if supercritical CO_2 extraction technology wants to be used with wheat bran. After the extracts were put through a process of centrifugation, three clearly visible phases became present. A watery phase, result of the moisture in the bran and partly because of the extraction process, a waxy phase, rich in waxy esters and a source for some interesting molecules such as alkylresorcinols, and an oily phase. The presence of the mentioned waxy esters and alkylresorcinols most probably is the clue to the low yields obtained during this experiment.

One of the most worrying characteristics of wheat bran oil extracted with this type of technology and once it is refined, is the high presence in it of products of fatty acid hydrolysis. Running a thin-layer-chromatography analysis with the wheat bran oil obtained with supercritical CO_2 technology revealed that it contained very high amounts of free fatty

acids. This hydrolysis was not of enzymatic origin, since another type of bran was then started to be used in this research, which had been pre-treated to deactivate enzymes such as the lipases. The same analysis was done on oils extracted with hexane and the soxhlet method. Free fatty acids were still present in those samples but the levels were significantly lower. A probable reason for the high levels of hydrolysis in the CO_2 extracted samples could be the high pressure levels (450 bar or higher) achieved during the extraction process with this technology.

Even taking into account the aforementioned problems that appeared when trying to apply this technology to the extraction of wheat bran oils, it is to be said that these had an interesting fatty acid profile. Polyunsaturated fatty acids were by far the most abundant (56%), especially linoleic acid (51%). The oil also contains an interesting amount of monounsaturated fatty acids such as oleic acid (12%) and the most abundant saturated fatty acid is palmitic acid (15%). These high levels of unsaturation, which is considered to have a positive impact in human health against cardiovascular disease and other problems, and the relatively low amounts of saturation open a new dimension for wheat bran oils in the fields of food fortification and dietetics. The total amount of fatty acids in each sample was around 86%. The remaining percentage is due to the presence of other substances such as hydrolysis subproducts (free fatty acids) or rests of waxy esters and water due to an imperfect raffination.

The oils were also analysed in search of certain substances of interest such as sterols and stanols. Sterols and stanols have interest in human nutrition since they have the capacity to lower LDL and plasma cholesterol, because of their similar structure to these molecules and this has been demonstrated to drastically reduce morbidity and mortality. Our samples were found to contain important amounts of plant sterols and stanols in amounts that were hanging about the 2 g every 100 g of oil sample. Almost half of that amount was found to be β- sitosterol, which was by far the most abundant of all, followed by stigmasterol, stigmastanol and sitostanol. It can be therefore stated that wheat bran oil could have an interesting use as a nutritional source for phytosterols and phytostanols and have a positive impact on human health. The highest yields of sterols and stanols were obtained at the highest temperatures used in our experiments (60 ºC) as well as the highest sample moisture (13%), whilst pressure seemed to have its highest impact at moderate values (around 350 bar).

Another type of interesting substances that can be found in wheat bran oil is the tocol group (vitamin E family of compounds), which comprises tocopherols, and tocotrienols, all of which have significant antioxidant properties. Around 150 mg of tocols could be found every 100 g of oil sample. Among all the tocols identified, β-tocotrienol was by far the most

common (90 mg/100 g oil) followed by α and β-tocopherol (25 mg /100 g oil aprox.) and α-tocotrienol (15 mg / 100 g of oil). The amounts of α- and β-tocopherol were significantly lower to some found in the literature, which had been obtained at similar extraction conditions. Extraction parameters did have a slight impact on the yields of these substances. Temperature had a positive impact and the best results were obtained with a combination of a temperature of 60 ºC and a pressure of 450 bars. Comparing both sorts of wheat bran used in this research, brings out that the special food bran had slightly higher tocol contents than the normal bran but it does not seem likely that the special treatment with steam that the food bran receives to de-activate certain enzymes could have an impact in the tocol yields of the oil. Considering that the supplier and wheat sort are the same, these minor differences are more likely to be a result of both brans coming from different harvests of wheat from different times of the year.

One of the reasons in favour to try to find a way for these kind of bran extractions into the industry is that they offer not only a valuable extract rich in certain antioxidants and substances with positive health effects such as the aforementioned sterols, stanols, tocotrienols or tocopherols but also a very valuable by-product that is the defatted bran. This by-product is rich in dietary fibre and protein and low in fat, and moreover it contains no traces of toxic extraction elements. On these grounds, the defatted bran could easily find a way into the animal feed market as a high value nutritional product.

Summarizing, wheat bran oil can be an interesting product with commercial applications, such as a source of antioxidants for cosmetic industry, or as complement to human diet due to its high abundance in polyunsaturated fatty acids and substances as tocols, sterols and stanols, but supercritical CO_2 is probably not the most convenient technology to obtain it since several issues appeared during and after the extraction due to its characteristics.

7 Summary

Supercritical CO_2 extraction is an innovative type of extraction that has been successfully used in many occasions to extract oils, flavours and other compounds out of a wide range of raw materials. One of the most remarkable advantages of this type of extraction is that, unlike other organic solvents used commonly in the industry, CO_2 is a non-toxic substance that leaves no traces in the extract or the by-product, since it gets evaporated soon after the extraction is finished. Its main disadvantages nowadays are the efficiency and price of the equipment, as well as the impossibility to perform extractions in a continuous manner, which hinders its possibilities to find an application in the industry. Wheat bran oil can be an interesting product with commercial applications, such as a source of antioxidants for cosmetic industry, or as complement to human diet due to its high abundance in polyunsaturated fatty acids (56%) and substances as tocopherols and tocotrienols (15 mg/100 g oil), sterols and stanols (2 g/100 g oil). Moreover, together with the oil, some by-products are obtained, such as a waxy phase rich in alkylresorcinols and defatted bran that can be used as a high-value feed source for animals, rich in fibre and protein and low in fat. However, this technology may not be the most convenient to obtain wheat bran oil since several issues appeared during and after the extraction, mostly due to some fractions of the extract remaining stuck to the walls of the separator making it impossible for them to be collected and making the oil yields too inconsistent to allow a detailed analysis of them.

8 Zusammenfassung

Extraktionen mit überkritischem CO_2 sind eine innovative Art der Extraktion, die sehr oft und mit Erfolg dazu gedient haben, Öle, Aromen und andere Verbindungen aus einer breiten Palette an Rohstoffen zu gewinnen. Einer der bedeutendsten Vorteile dieser Art der Extrahierung ist, dass, im Gegensatz zu anderen organischen Lösungsmitteln, die normalerweise in der Industrie verwendet werden, CO_2 eine nicht-toxische Substanz ist, die keine Spuren in dem Extrakt oder dem Nebenprodukt hinterlässt, da sie kurz nachdem die Extraktion beendet ist, verdampft. Die Hauptnachteile (größten Nachteile) sind heute die Effizienz und der Preis der Geräte, sowie die Unmöglichkeit die Extraktion in kontinuierlicher Weise durchzuführen. Dadurch werden die Möglichkeiten eine Anwendung in der Industrie zu finden gehindert. Weizenkleieöl kann ein interessantes Produkt für die kommerzielle Nutzung darstellen. Es kann zum Beispiel als eine Quelle von Antioxidantien für die kosmetische Industrie oder als eine Ergänzung für die menschliche Ernährung aufgrund seines hohen Anteils an mehrfach ungesättigten Fettsäuren (56%) und Substanzen wie Tocopherole und Tocotrienole (15 mg/100 g Öl), Sterole und Stanole (2 g/100 g Öl) genützt werden. Darüber hinaus werden, zusammen mit dem Öl, andere Nebenprodukte gewonnen, wie bei einer wachsartigen Phase reich an Alkylresorcinolen und entfettete Kleie. Die entfettete Kleie kann auch als eine hochwertige Futterquelle für Tiere, die reich an Ballatstoffen und Eiweiß ist und wenig Fett enthält, verwendet werden. Jedoch ist diese Technik nicht unbedingt die geeigneste um Weizenkleieöl zu erhalten, da mehrere Probleme während und nach der Extraktion entstanden sind, vor allem aufgrund einiger Bruchteile des Extraktes, die an den Wänden des Separators stecken geblieben sind. Es war nicht möglich diese zu sammeln und das gewonnene Öl wurde dadurch zu inkonsistent, um eine detaillierte Analyse von ihnen zu ermöglichen.

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