KAUNAS UNIVERSITY OF TECHNOLOGY

DARIUS POVILAITIS

BIOREFINING THE BY-PRODUCTS OF PLANT MATERIAL PROCESSING INTO VALUABLE FUNCTIONAL INGREDIENTS BY APPLYING VARIOUS EXTRACTION TECHNIQUES

Doctoral Dissertation
Technological Sciences, Chemical Engineering (T05)

2016, Kaunas

UDK 542.61+634.723+633.1](043.3)

The research was carried out at Kaunas University of Technology, Faculty of Chemical Technology, Department of Food Science and Technology during the years 2012–1016. The studies were supported by Research Council of Lithuania

Scientific supervisor:

Prof. Dr. Petras Rimantas Venskutonis (Kaunas University of Technology, Technological Sciences, Chemical Engineering – 05T).

Doctoral dissertation has been published in:
http://ktu.edu

English Language Editor:
Dovilė Dumbrauskaitė
Publishing house "Technologija"

ISBN 978-609-02-1281-3

KAUNO TECHNOLOGIJOS UNIVERSITETAS

DARIUS POVILAITIS

ŠALUTINIŲ AUGALINĖS ŽALIAVOS PERDIRBIMO PRODUKTŲ BIORAFINAVIMAS Į VERTINGUS FUNKCIONALIUOSIUS KOMPONENTUS TAIKANT ĮVAIRIUS EKSTRAKCIJOS METODUS

Daktaro disertacija
Technologijos mokslai, Chemijos inžinerija (05T)

2016, Kaunas

UDK 542.61+634.723+633.1](043.3)

Disertacija parengta 2012 – 2016 metais Kauno technologijos universitete, Cheminės technologijos fakultete, Maisto mokslo ir technologijos katedroje. Mokslinius tyrimus rėmė Lietuvos mokslo taryba.

Mokslinis vadovas:

Prof. Dr. Petras Rimantas Venskutonis (Kauno technologijos universitetas, technologiniai mokslai, chemijos inžinerija – 05T).

Interneto svetainės, kurioje skelbiama disertacija, adresas:
http://ktu.edu

Anglų kalbos redaktorė:
Dovilė Dumbrauskaitė
(Leidykla "Technologija")

ISBN 978-609-02-1281-3

AKNOWLEGMENTS

I would like to thank all the people who contributed or were involved in any part of this research.

I would like to express my gratitude to my scientific supervisor and advisor Prof. Dr. Petras Rimantas Venskutonis for his continuous support during research and kind help with article and thesis writing. Valuable discussions and innovative ideas were really helpful in my research path.

I am grateful to all the colleagues in the Department of Food Science and Technology for all the help during experiments, the analysis of results and friendly advice.

I want to thank my colleagues from the "basement lab" for all the help, stimulating discussions, and warm and cozy atmosphere during all my study years.

I am very grateful to my wonderful wife Jovita for her love, care and patience during these years. Last but not least, I want to thank my parents for their support and encouragement throughout my studies.

Thank you all for the support and encouragement!

Darius Povilaitis

LIST OF FIGURES

Figure 1. Thesis research scheme 16
Figure 1.1. Potential products made of food supply chain side products 18
Figure 1.2. Typical phase diagram of a pure solvent 20
Figure 1.3. The dielectric constant of water as a function of temperature at constant pressure 22
Figure 1.4. The three different extraction periods and the extraction curve 24
Figure 1.5. Central composite designs for the optimization of two and three variables 27
Figure 1.6. Sources of reactive oxygen species 29
Figure 1.7. The peroxidation of LH initiated by X radical and inhibited by antioxidant QH_2 30
Figure 1.8. Resonance stabilization of a free radical by a phenolic 31
Figure 1.9. Chemical structures of different classes of polyphenols and sub-classes of flavonoids in plants 33
Figure 1.10. The chemical structure of the $ABTS^{\bullet+}$ radical 35
Figure 1.11. The chemical structure of the $DPPH^{\bullet}$ radical 36
Figure 1.12. The reduction reaction of FRAP assay 36
Figure 1.13. Longitudinal and cross sections of a wheat kernel 38
Figure 1.14. A scanning electron micrograph of a cross section of a hard winter wheat kernel 38
Figure 1.15. The structures of major phytochemicals extracted from various cereal brans 39
Figure 1.16. A scanning electron micrograph of the outer part of a rye kernel 42
Figure 1.17. 5-n-alkylresorcinols found in rye bran 43
Figure 2.1. The principle schematic diagram of SC-CO_2 system 48
Figure 3.1. The accumulative antioxidant activity values of rye and wheat bran 61
Figure 3.2. The correlation coefficients between different antioxidant measurement assays of different rye and wheat bran milling fractions 62
Figure 3.3. The biorefining scheme of rye bran by SC-CO_2 and PLE 63
Figure 3.4. The influence of dynamic extraction time on the rye bran extract yield 64
Figure 3.5. Response surface plots for the effects of SC-CO_2 for the yield of rye bran oil 67
Figure 3.6. A comparison of predicted values and actual values for the extraction yield of rye bran 68
Figure 3.7. Response surface plots for the effects of PLE with acetone for the yield of rye bran extract from residue after SC-CO_2. 72
Figure 3.8. Response surface plots for total phenolic content in acetone extracts 73
Figure 3.9. The biorefining scheme of blackcurrant pomace by SC-CO_2, PLE and EAE 75
Figure 3.10. Response surface plots for the effects of SC-CO_2 on the extract yield of blackcurrant pomace 78
Figure 3.11. The kinetics of blackcurrant pomace SC-CO_2 extraction at optimal extraction parameters 79

Figure 3.12. A comparison of actual and predicted extract yield from SC-CO_2 extraction of blackcurrant pomace 80
Figure 3.13. The blackcurrant extract and residues obtained from different extractions..... 81
Figure 3.14. The oxidation curves of blackcurrant pomace oil isolated by SC-CO_2 and selected commercial oils. 84

LIST OF TABLES

Table 2.1. Independent variables and their variation levels for the central composite design..... 53
Table 3.1. The extraction yield of wheat and rye bran obtained by PLE..... 56
Table 3.2. The results of rye and wheat bran antioxidant activity. 58
Table 3.3. CCD parameter values and the results of rye bran extract optimization. 65
Table 3.4. ANOVA for evaluated response surface quadratic model for rye bran .. 68
Table 3.5. The fatty acid composition of rye bran oil extracted by SC-CO_2 70
Table 3.6. Parameter values and results of rye bran PLE with acetone optimization..... 71
Table 3.7. Analysis of variance for the response surface quadratic model..... 72
Table 3.8. The antioxidant characteristics of solid substances obtained after SC-CO_2 extraction and PLE 74
Table 3.9. The CCD parameter values and results of blackcurrant pomace extract optimization..... 77
Table 3.10. ANOVA for evaluated response surface quadratic model for blackcurrant pomace..... 80
Table 3.11. Yields of soluble fractions of blackcurrant pomace after enzymatic treatment..... 82
Table 3.12. The composition of fatty acids (g/100 g) in pomace oil extracted by SC-CO_2 and Soxhlet 83
Table 3.13. Total phenolic content and the antioxidant properties of extracts from Soxhlet extraction, SC-CO_2 and PLE 85
Table 3.14. The antioxidant characteristics of soluble substances (extracts) obtained after EAE of blackcurrant pomace and corresponding control samples prior and after SC-CO_2, and after PLE-EtOH 86
Table 3.15. The effect of enzymes on the antioxidant characteristics of solid substances obtained after EAE of blackcurrant pomace and corresponding control samples prior and after SC-CO_2, and after PLE-EtOH..... 87

CONTENTS

CONTENTS 9
ABREVIATIONS 11
INTRODUCTION 13
1. LITERATURE REVIEW 17
1.1. The concept of biorefining 17
1.2. The extraction of bioactive compounds 18
1.2.1. High pressure extraction techniques 19
1.2.2. Extraction using enzymes 25
1.3. Extraction process optimization 26
1.4. Nutrition and antioxidants 28
1.4.1. The mechanism of lipid oxidation 29
1.4.2. Antioxidants 32
1.4.3. Methods for determining antioxidant activity 33
1.5. The characterization of selected cereal and berry processing by-products 37
1.5.1. Cereal grains 37
1.5.2. Blackcurrant 44
2. MATERIALS AND METHODS 45
2.1. Research objects and pretreatment 45
2.1.1. Reagents 46
2.2. The preparation of extracts 46
2.2.1. Pressurized liquid extraction 47
2.2.2. Supercritical carbon dioxide extraction 48
2.2.3. Enzyme-assisted extraction 49
2.3. The evaluation of antioxidant activity 49
2.3.1. Oxygen radical absorbance capacity assay 49
2.3.2. Total phenolic content by Folin-Ciocalteu's assay 50
2.3.3. $ABTS^{\bullet+}$ decolourization assay 51
2.3.4. $DPPH^{\bullet}$ scavenging assay 51
2.3.5. Ferric reducing antioxidant power assay 52
2.3.5. The evaluation of oil stability 52
2.4. Chromatographic analysis 52
2.4.1 The determination of fatty acid profile by gas chromatography 52
2.4.2 High performance liquid chromatographic determination of tocopherols 53

2.5. The experimental design 53
2.6. Statistical data evaluation 55
3. RESULTS AND DISCUSSION 55
3.1. The extraction yield and antioxidant properties of wheat and rye bran extracts obtained with pressurized liquid extraction 55
3.1.1. The extraction yield of wheat and rye bran 55
3.1.2. The effect of solvent on antioxidant activity indicators of rye and wheat bran extracts 56
3.1.3. The effect of particle size on antioxidant activity indicators of rye and wheat bran extracts 59
3.1.4. The overall antioxidant activity and correlation between different determination methods 60
3.2. The optimization of high pressure extraction of rye bran using mathematic modelling tools 63
3.2.1 The optimization of the supercritical fluid extraction of rye bran 64
3.2.2 A statistical analysis of rye bran extraction optimization model 67
3.2.3. The fatty acid composition and antioxidant capacity of rye bran oil 69
3.2.4. The optimization of rye bran residue extraction using pressurized liquid extraction 71
3.2.5. The antioxidant activity of rye bran fractions obtained by PLE 73
3.3. Biorefining of blackcurrant pomace into high value ingredients 75
3.3.1. The optimization of supercritical fluid extraction parameters 76
3.3.2 The analysis of blackcurrant pomace supercritical fluid extraction optimization model 79
3.3.3. Pressurized liquid and enzyme-assisted extraction of pomace 80
3.3.4. The analysis of fatty acid, tocopherols and oil stability of extract 82
3.3.5. TPC and antioxidant activity of extracts and solid residues 84
CONCLUSIONS 88
REFERENCES 90
LIST OF PUBLICATIONS ON THE TOPIC OF DISSERTATION 102

ABREVIATIONS

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride;
$ABTS^{\bullet+}$	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid);
ANOVA	Analysis of variance;
AOA	Antioxidant activity;
CCD	Central composite design;
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical;
DW	dry sample weight;
E/S	Enzyme substrate ratio;
EAE	Enzyme-assisted extraction;
EDW	Extract dry weight;
EtOH	Ethanol;
FA	Fatty acid;
FC	Folin-Ciocalteu;
FRAP	Ferric reducing antioxidant power;
GAE	Gallic acid equivalent;
HPLC	High performance liquid chromatography;
L-ORAC	Oxygen radical antioxidant capacity for lipophilic compounds;
MeOH	Methanol;
ORAC	Oxygen radical absorbance capacity;
PLE	Pressurized liquid extraction;
QUENCHER	Quick, easy, new, cheap and reproducible;
RB	Rye bran;
ROS	Reactive oxygen species;
RSC	Radial scavenging capacity;
RSD	Relative standard deviation;
RSM	Response surface methodology;
SC-CO_2	Supercritical carbon dioxide;
SD	Standard deviation;
SFE	Supercritical fluid extraction;

TE	Trolox equivalent;
TEAC	Trolox equivalent antioxidant capacity;
TPC	Total phenolic content;
WB	Wheat bran.

INTRODUCTION

There is a growing demand for healthy food products, since more and more people are concerned about their health. Consequently, many researchers are searching for new, natural and valuable bioactive compounds. Finding new, safe and effective antioxidants from natural sources is an important task, since their beneficial effect on human health is well known. Furthermore, it is crucial to reduce the growing production of industrial waste. Combining novel plant biomass conversion technologies allows to decrease by-product production and develop new promising value-added products and chemicals (Ravindran & Jaiswal, 2015).

Cereal bran is an often inefficiently used by-product from flour milling, although it can be a great source for the extraction of valuable compounds. It is the nutritional storehouse of grains and its resources can be used to enrich our diet with various bioactive compounds, such as dietary fibers, essential fatty acids, phytosterols, various antioxidants, minerals, vitamins and proteins (Patel, 2012). Even though it is assumed that the entire wheat grain is used for flour production, about 175 million tons of bran is produced every year. Since rye grain is mainly used in the Nordic countries, the amount of rye bran is lower and comprises about 3.75 million tons. Fruit pomaces can also be a great stock of various bioactive compounds. The production of different juices from berries results in high amounts of valuable residue. This by-product is a rich source of food ingredients and functional constitutes which can be applied to enhance the functionality of food products. Usually, positive effects on health are visible when sufficient amounts of required components are consumed. However, it is difficult to obtain this quantity through consumption of fresh fruit or common commercial products, thus food additives that contain sufficient amount of the compounds which have a positive effect on health can be prepared by using different by-product processing techniques (Galanakis, 2012a).

In order to successfully obtain valuable compounds, it is best to consecutively employ different extraction technologies. One of the possibilities is to use high pressure extraction technologies which are very efficient, fairly easy to handle and usually require less harmful solvents. The best results can be achieved by applying these technologies accompanied by various mathematical optimization techniques.

This study investigates the valorization of rye and wheat bran and blackcurrant pomace using multistep processes for biorefining the biomass into high value food-grade functional ingredients. Firstly, rye and wheat bran antioxidant activity was evaluated by using pressurized solvent extraction with organic solvents. Then, environmentally friendly supercritical carbon dioxide extraction was optimized using response surface methodology with central composite design for effective isolation of lipophilic fraction. Finally, pressurized liquid extraction and enzyme-assisted extractions were applied to isolate higher polarity substances. Different antioxidant activity assays were applied to determine the best fractions for the value-added ingredient production.

Aim of the thesis

The aim of the thesis is to develop effective schemes for biorefining cereal bran and blackcurrant pomace into valuable functional ingredients by using effective high pressure extraction/fractionation and enzymatic treatment techniques.

Objectives of the thesis are:

1. To evaluate the influence of extraction solvent and particle size on the recovery of rye and wheat bran extract.

2. To evaluate the influence of extraction solvent and particle size on the antioxidant properties of rye and wheat bran extracts.

3. To optimize the process of supercritical carbon dioxide extraction for isolating lipophilic fraction from rye bran and to evaluate the properties of the obtained extracts.

4. To evaluate the possibilities of rye bran fractionation using pressurized liquid extraction technologies and extract characterization. To optimize the process of pressurized liquid extraction with acetone.

5. To develop a scheme for biorefining blackcurrant pomace by optimizing high pressure and enzyme-assisted extraction parameters.

6. To evaluate the properties of blackcurrant pomace extract and to explore the possibility to use it as food supplement or additive.

Scientific novelty

1. Pressurized liquid extraction has been applied and comprehensively evaluated for the isolation of bioactive substances from rye and wheat bran for the first time. Moreover, the scientific knowledge on rye bran extraction, fractionation and characterization of the products obtained has been substantially expanded in general, since so far the processing of rye bran has been remarkably less studied than that of wheat bran.

2. The optimization of supercritical carbon dioxide extraction parameters for isolating the highest yields of lipophilic fraction from rye bran has not been performed before this research.

3. This research pioneers in optimizing the supercritical carbon dioxide extraction parameters for isolating lipidic fraction from blackcurrant pomace and developing a scheme combining pressurized liquid and enzyme-assisted extraction for biorefining blackcurrant pomace into valuable functional ingredients.

The practical value of the work

During flour milling or various fruit juice production large amounts of agro product waste, such as cereal bran and berry pomaces are accumulated from different production lines. These materials are usually discarded (mainly berry pomace) or used rather inefficiently, although they can be a rich source of valuable components. By applying novel extraction technologies and biorefining, these

unexploited materials can be transformed into very beneficial products. Since every material has a unique biological and chemical structure, it is very important to evaluate possible applications and extraction methods individually. By applying high pressure extraction technologies alongside with mathematical modeling tools, it is possible to achieve high efficiency in fractionation and recover valuable compounds. To the best of our knowledge, no existent experiments have analyzed the composition of rye and wheat bran antioxidants by pressurized liquid extraction applying different solvents. Furthermore, there have not been any experiments optimizing supercritical carbon dioxide extraction of rye bran or blackcurrant pomace. Furthermore, this is the first time that a combination of supercritical carbon dioxide and pressurized liquid extraction is used on these materials. The most important variables have been optimized in order to achieve the highest efficiency of the applied process. By consecutively applying these methods almost all valuable components can be extracted from the analyzed material. After extraction, the residue can be used for biofuel production. The results obtained on laboratory equipment may be applied for upscaling the processes to pilot plant and finally industrial levels. Consequently, the results of the study may be considered as the first and important step for the commercialization of the developed biorefining schemes.

Structure and outline of the dissertation

This dissertation is divided into the following parts: the list of abbreviations, the introduction, the literature review, materials and methods, results and discussion, conclusions, a list of 164 references and a list of publications on the topic of the dissertation. The literature overview and results of the research are presented in 104 pages, including 33 figures and 16 tables.

Publication of the research results

The results of this research are presented in two publications, corresponding to the list of the Thompson Reuters Web of Science database, and reported at 6 international conferences.

Statements presented for defense of the dissertation

1. High performance extraction techniques, such as pressurized liquid extraction and supercritical carbon dioxide extraction, may be efficiently applied for isolating valuable substances from rye and wheat bran; whereas response surface methodology using central composite design may provide reliable mathematic models for the optimization of extraction parameters.

2. Selecting combined high pressure and enzyme-assisted extraction methods and schemes as well as optimization of their parameters may provide efficient blackcurrant pomace biorefining technology for obtaining high value bioactive substances.

Thesis research scheme

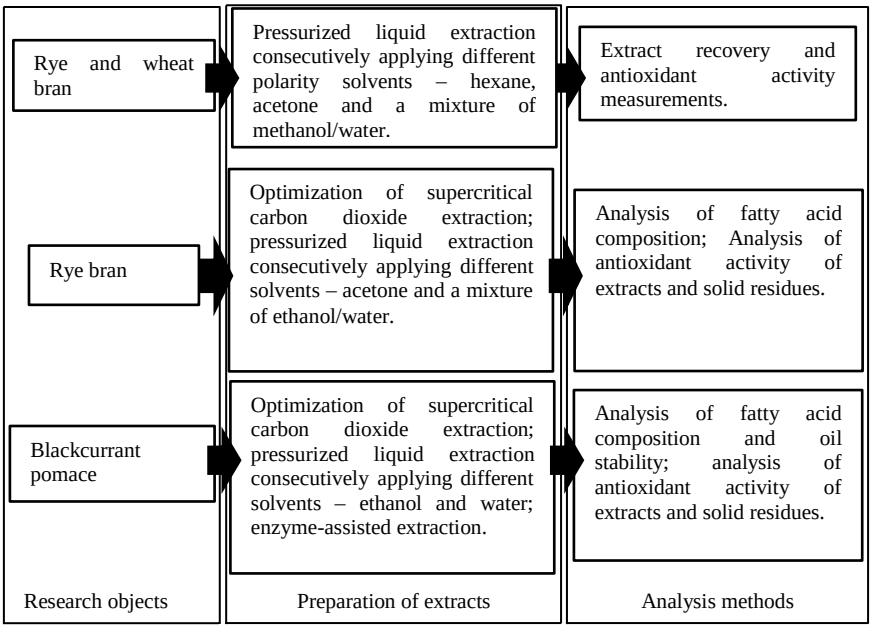


Figure 1. Thesis research scheme

1. LITERATURE REVIEW

1.1. The concept of biorefining

The population of the world increases every year and the food sources are becoming insufficient to meet the needs of the society. It is very important to use the natural resources wisely since they are slowly decreasing. A great portion of worldwide material products comes from refining fossil fuels, but it is predicted that the feasibility of oil exploitation will decrease in the near future. In order to minimize our impact on Earth climate change, the reduction of fossil fuel consumption should be promoted. Oil can be replaced with biomass as raw material for fuel and chemical production. Using jointly applied novel conversion technologies almost all types of biomass feedstock can be converted into different classes of biofuels and biochemicals.

Biorefining can be explained as sustainable processing of biomass into a spectrum of marketable products and energy. It comprises of a wide range of technologies able to separate the biomass resources (grasses, grains, food waste) into their building blocks (carbohydrates, proteins, triglycerides) which can be converted into value-added products and chemicals. This concept is very similar to today's petroleum refinery, where different valuable products are produced from crude oil (Cherubini, 2010).

According to the Food and Agriculture Organization (FAO), about one-third of the edible parts of food produced for human consumption gets lost or wasted globally. This amount is huge (1.3 billion tons per year) and reflects food processing wastes and food losses during production, postharvest, handling, processing, distribution and consumption stages (Galanakis, 2012b; Gustavsson, Cederberg, & Sonesson, 2011). A study published by the EU in 2010 estimates that almost 90 million tons of food waste are discarded from food manufacturing industry every year (European Commission, 2010). This organic material is high in nutritional value so it putrefies when it is accumulated. Consequently, it is a good place for disease-causing organisms which can produce serious environmental problems. Preventive actions can be taken to minimize this waste accumulation but it is important to deal with the existing food waste. Converting food waste into energy and other useful chemicals or substances used in our daily life is an area of research with high potential (Ravindran & Jaiswal, 2015).

Food wastes are composed of complex constituents and they can be isolated adapting different extraction, fractionation and isolation technologies. Basically all food wastes can be classified into two main groups by their origin: plant-derived and animal-derived wastes (**Fig. 1.1**). Figure 1.1 shows the main materials which are extracted from the by-products of food processing. The main focus is on the recovery of functional compounds derived from agricultural and food processing by-products. Collecting them requires less effort, they are more concentrated and less susceptible to deterioration in comparison to the waste collected at the end of food supply chain. It would be difficult to collect them across a broad range of households and their composition may highly vary. The valorization is complicated because an additional step is needed to collect the waste and corresponding

biological stability is dramatically reduced due to the growth of pathogens (Galanakis, 2012b).

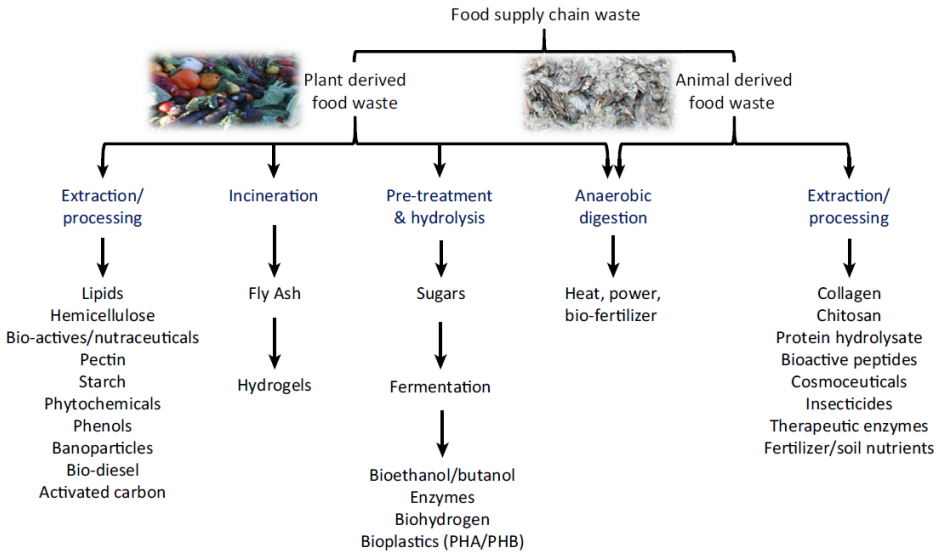


Figure 1.1. Potential products made of food supply chain side products (Ravindran & Jaiswal, 2015)

Cereals are very widely spread around the world and the dominant crop is wheat (in medium- and high-income countries). Rice grain is more popular in South and Southeast Asia (Gustavsson et al., 2011). Flour milling or rice husking generates by-products like straw or bran, which are a rich source of healthy phytochemicals (phenolic, flavonoids, glucans and pigments). Numerous recent studies have indicated that consuming more whole grain foods can lead to improved health, reduce the risk of obesity, diabetes, coronary troubles and cancer (Patel, 2012). The wastes of fruit and vegetables processing are the most widely analyzed substrates for the extraction of antioxidants and dietary fiber. These products remain after juice production and they consist of soft tissues that are rich in valuable ingredients (Galanakis, 2012b).

1.2. The extraction of bioactive compounds

Different plants consist of complex ingredients which may be in a wide range of polarity and hydrophobicity. All these compounds can be divided into several groups by their polarity: low-polar (wax, terpenoids), semi-polar (lipids, phenolic) and highly-polar (polar glycosides, polar alkaloids, saccharides, peptides and proteins) (Romanik et al., 2007).

Essentially all sample preparation procedures for the composition analysis consist of three main steps:

Sample preparation is necessary to homogenize the examined material. Different sample preparation techniques can be used; however, drying, freeze-drying and milling are applied most commonly. These processes allow a higher diffusion of

extracts inside the food matrix. Thermal pretreatment (drying at high temperatures) can lead to a loss of functionality, while freeze-drying can increase the cost due to the presence of vacuum conditions. Centrifugation and microfiltration have also been suggested because these techniques enable the removal of solids, oils and fats. Furthermore, extracting the components with specific properties requires a more complicated approach during pretreatment (e.g. hydrolysis, saponification) (Galanakis, 2012b).

Extraction of valuable components is perhaps the most important stage; different methodologies can be employed depending on the target molecules and their physicochemical characteristics. Solvent extraction is convenient because it provides a physical carrier of target molecules between different physical phases (solid, liquid and vapor). The range of extracted components depends on the solvent and conditions of the extraction process. For example, phenols are easily soluble in hydroalcoholic mixtures, while different fractions may be obtained on the basis of polarity by varying alcohol concentrations (Galanakis et al., 2011). Ethanol is mostly preferred for alcoholic extraction because it is comparatively cheap and has the "GRAS" (Generally-Recognized-As-Safe, according to American Food and Drug administration) status. However, sometimes it is necessary to use other solvents (acetone, hexane or others) to obtain lipophilic, non-polar compounds. In such instances, the solvent should be removed from the extract completely before it can be used in food products. Occasionally, solvents are used in combination with pressure which accelerates the process of extraction by enhancing solvent penetration through the sample matrix. Furthermore, sequential extraction steps increase the extract yield but prolongs the extraction process which might increase the cost of the process (Galanakis, 2012b).

Analyte enrichment is often included, although sometimes not required in sample preparation. It is performed simultaneously with removing interferences caused by such techniques as liquid/liquid extraction, solid-phase extraction, selective adsorption, preparative liquid chromatography (with different stationary phases), ion chromatography, size-exclusion chromatography, solid-phase micro-extraction and solvent micro-extraction.

When all these steps are performed, the sample can be subjected to final separation and analysis. The final analysis can be performed by using various chromatography techniques, such as liquid or gas chromatography. Antioxidant activity assays can be also applied to measure the antioxidant properties of the sample. Firstly, a qualitative analysis has to be performed to identify the selected or all components. Secondly, the sample can be analyzed quantitatively, followed by the calibration of the instrument. Performing both qualitative and quantitative analyses simultaneously is less time-consuming (Romanik et al., 2007).

1.2.1. High pressure extraction techniques

A lot of effort has been devoted to the development of more efficient extraction techniques in the last two decades. Consequently, systems that are operating under medium-to-high pressure have been invented. Due to physicochemical properties of the solvent, pressurized solvent techniques enhance the target molecule specificity

and velocity. Varying the pressure and temperature of the extraction system provides the ability to control solvent density, diffusivity, viscosity and dielectric constant (Wijngaard et al., 2012). There are many methods applying high pressure extraction, two of which are widely spread. Supercritical fluid extraction (SFE) usually uses carbon dioxide (SC-CO_2), while pressurized liquid extraction (PLE) is popular because it can be easily adapted for different solvents. The latter technique is frequently used with water at subcritical conditions, which is known as subcritical water extraction (SWE) (Pronyk & Mazza, 2009).

Extraction fluids reach supercritical fluid state when they are subjected to a pressure and temperature above their critical point (**Fig. 1.2**) (Herrero, Cifuentes, & Ibanez, 2006). The main difference between SFE and PLE is the solvent state during extraction. SFE is based on the use of solvents at temperatures and pressures above their critical points, while PLE operates using liquids at temperatures above their boiling points. In the pressurized liquid extractor pressures have to be high enough to keep the extracting fluid in the liquid state (Herrero et al., 2013). Using these techniques, the absence of light and air during the extraction reduces the risk of degradation processes that can occur using conventional extraction technologies (Bleve et al., 2008).

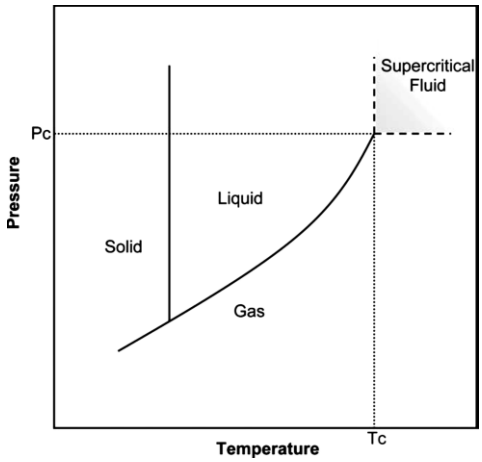


Figure 1.2. Typical phase diagram of a pure solvent (Herrero et al., 2006)

1.2.1.1. Pressurized liquid extraction

Pressurized liquid extraction is very similar to supercritical fluid extraction but it is operated at lower pressures. The main principle of this technique is to allow conventional solvents to be used at higher temperatures, keeping them under the boiling point. Thus, smaller volumes of solvent can be used and the extraction is faster. As in the SFE application, it is important to optimize different factors influencing the outcome of the extraction process. This has to be done for each particular sample because each sample has a unique chemical and morphological structure. The most important parameter in the extraction procedure is temperature. If the temperature is kept high enough, the expected yield will be the highest. However, when working with bioactive compounds, the temperature has to be

closely examined and optimized. High temperatures have a negative effect on the bioactivity of some thermo-labile compounds that can degrade during extraction. Even if there are some experiments which suggest that high temperatures can be applied for bioactive compound extraction without any loss of the compounds, this parameter has to be studied for each type of matrix and each valuable compound. However, when high temperatures are applied, new components might form in the extracts. They can interfere with natural plant components and skew the results. This might be useful when searching for new valuable components but it causes problems when the natural composition of the sample has to be analyzed.

It is important to keep the extraction pressure high enough to maintain the solvents in the liquid state. Previous works report that pressure has a very small or even no effect on the extraction efficiency (Wijngaard et al., 2012). The extraction time depends on what mode of extraction is applied. There are two possible modes: static and dynamic. In static mode, a certain amount of solvent is kept in contact with the sample material for a given period of time under the desired pressure and temperature. In static mode of extraction, sample components which are still bound to the sample may reach equilibrium with those already solubilized in the solvent. To solve this shortcoming, several consecutive static extraction cycles can be used. For some applications one extraction cycle is sufficient, while others may require 3 or more. When using the dynamic mode, fresh solvent is continuously introduced into the extraction cell. Using this procedure, the equilibrium might be delayed and the efficiency increases. This extraction mode provides higher recovery than static extraction but it may require more solvent (Herrero et al., 2013).

Solvents which are used in conventional extraction techniques can be applied in PLE. Ethanol, water or their mixtures are the most commonly used in PLE extractions. While "GRAS" solvents are preferred, several more toxic and harmful solvents (e.g. petroleum ether, dichloromethane) have also been used for the extraction of target compounds. A prominent trend nowadays is applying new environmentally friendly and food-grade solvents, such as water at subcritical conditions for subcritical water extraction (SWE). The increasing water temperature weakens the hydrogen bonds resulting in a lower dielectric constant (ε) which measures the solvent polarity; it highly varies when different water temperatures are used (**Fig. 1.3**). Water can reach values that are similar to less polar solvents at room temperature therefore it can be used as an alternative to dissolve medium-polar and even non-polar organic compounds (Herrero et al., 2013).

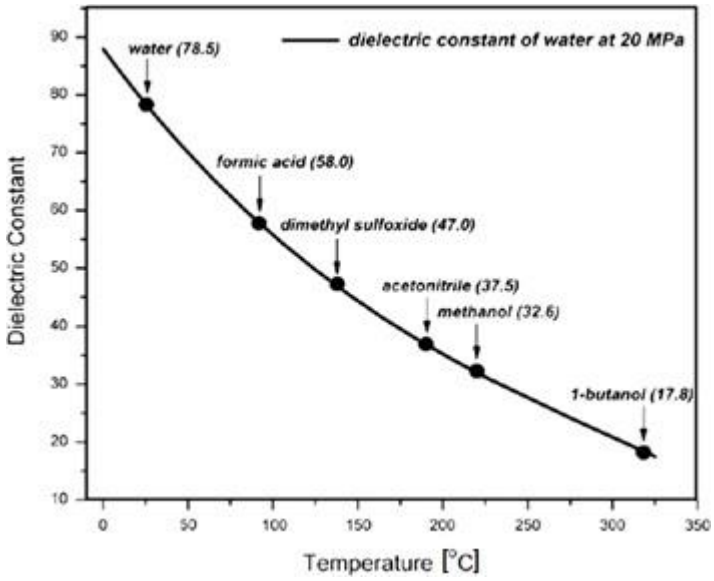


Figure 1.3. The dielectric constant of water as a function of temperature at constant pressure. Solid circles correspond to different organic solvents at room temperature and pressure (Herrero et al., 2013)

PLE can be used for the extraction of bioactive compounds from natural matrices and it has mainly been used to obtain antioxidants. Generally, commercial instruments work in static extraction mode for various extraction times (usually up to 20 min). Furthermore, it is common to re-extract the same sample multiple times consecutively. Normally, three extraction cycles are sufficient. Various temperatures (100−160 °C) have been widely applied to extract various bioactive compounds. PLE has several advantages over the more conventional techniques: it is more efficient, requires significantly less solvent, the extraction is faster and the process is automated.

PLE system can be coupled with other processes on-line (e.g. ultrasound assisted extraction or solid phase extraction) to increase the extraction efficiency and the purity of the compounds (Rostagno et al., 2010). The extraction yield can be increased by pretreating the in-cell enzyme before the extraction which releases the components from the natural matrix. Compounds of interest can be retained using in-cell solid phase extraction materials. This process can be improved by adding materials specifically designed to affect only the molecule of interest. A variety of approaches is available for isolating and purifying bioactive compounds (Herrero et al., 2013).

1.2.1.2. Supercritical fluid extraction

Supercritical fluid extraction (SFE) is a modern technique that involves the use of solvent above its critical temperature and pressure. In this state, liquid and gas are indistinguishable from each other. Supercritical fluids possess better transport properties than liquids and diffuse easily through solid materials therefore the extraction can be performed faster. The density of the fluid can be modified by

altering the extraction pressure and/or temperature which results in modified solvent power of the fluid; this technique is more efficient because it increases the yield and decreases the extraction time. The use of supercritical CO_2 as solvent produces solvent-free extracts. After the extraction is finished, the depressurization of the system turns carbon dioxide to gas while the extract precipitates. CO_2 is a low polarity solvent but by adding a small amount of polar modifiers its polarity can be changed, thus increasing solvation power towards the valuable components (Herrero et al., 2006).

Although there is a wide range of solvents that can be used as supercritical fluids (ethane, methanol, water, nitrous oxide, *n*-pentane and *n*-butene), carbon dioxide is the most popular because of its moderate critical temperature (31.1 °C) and pressure (7.39 MPa). At room temperature, carbon dioxide is a gas, so it easily evaporates from the obtained extract without performing solvent evaporation. This non-toxic, non-flammable solvent is considered as "GRAS" and can be used in the food industry. If the concentration of carbon dioxide in the extract is high, the extraction system can be adapted to recycle it.

SFE efficiency depends on several factors and it is important to consider each of them before every new extraction. The most important parameters (i.e. extraction pressure, temperature, addition, amount and type of modifier, amount of sample, particle size and extraction time) have to be calculated; all of these parameters are relevant in the present experiment. Pressure and temperature have a strong influence on the solvent density, which means that it greatly affects the solubility of target compounds. The supercritical fluid polarity can be changed by adding modifiers, such as ethanol, the concentration of which may vary in a range 5−10 % of CO_2 flow. Although ethanol is one of the most common modifiers, methanol, acetone and water can also be used to isolate polar compounds. Particle size and the use of dispersing agents are more related to the efficiency of the extraction procedure; these parameters can be altered to avoid the systems from clogging and to increase the rate of extraction. Particle size is often recognized as a significant factor in the mass-transfer rate, thus it can considerably influence the extract recovery.

The optimization of the SFE process requires knowledge of thermodynamic as well as kinetic data. The extraction curve of accumulated extract mass over extraction time can be used as kinetic data, which is normally represented with a graph. The curve is highly dependent on the process parameters (solvent flow and particle size) hence it is difficult to compare curves obtained from different raw materials. According to previous experiments, the overall extraction curve can be divided into three periods (**Fig. 1.4**), each presenting different mass transfer mechanisms. Firstly, constant extraction rate (*CER*) takes place; during this stage, the external surface of the particles is covered with solvent. The dominant mechanism of mass transfer is convection. Secondly, falling extraction rate (*FER*) period occurs; failures in the external surface oil layer appear and the diffusion mechanism begins. This step is combined with convection. Thirdly, low extraction rate (*LER*) or diffusion-controlled period (*DC*) is in process. The external layer of oil practically disappears and the mass transfer mechanism occurs mainly by diffusion

inside the solid particles of the sample matrix (Silva, Rocha-Santos, & Duarte, 2016).

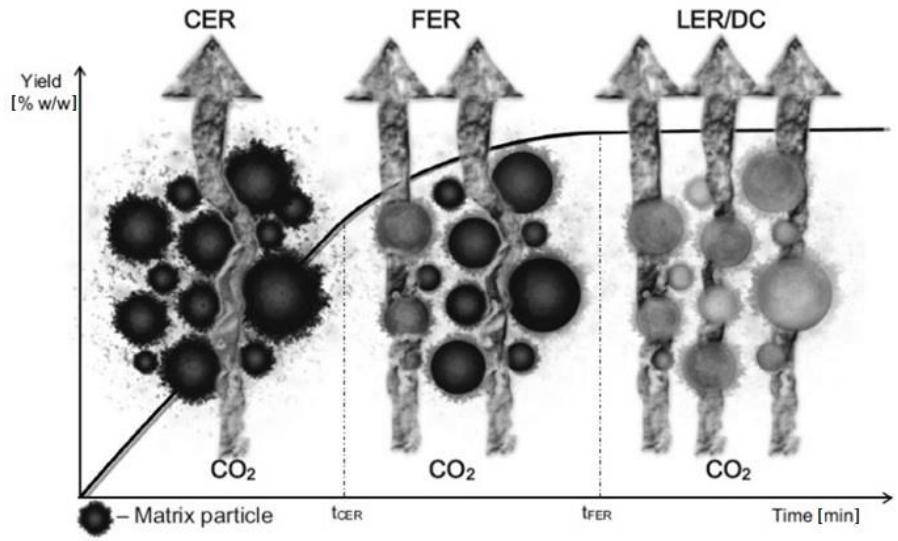


Figure 1.4. The three different extraction periods and the extraction curve (da Silva et al., 2016)

SFE equipment is mainly used to isolate bioactive non-polar compounds from various natural sources (e.g. plants, algae or microalgae and dairy products). Essential oil extraction is another important application of SFE. This equipment allows extracting valuable essential oils at low temperatures which preserves the integrity of the sample. Essential oils have a complex composition and besides their fragrance they can possess various bioactive properties (e.g. antimicrobial, antioxidant). Normally, pressures from 10 to 30 MPa and temperatures in the range of ~40−50 °C are used for various extractions (Herrero et al., 2013; Mendiola et al., 2007).

The design of supercritical fluid extraction system can be relatively simple or highly complex, depending on the purpose. Analytical systems are used to work with small sample quantities, to obtain milligrams to grams of extracts. Using these systems, the extraction experiments can be optimized and various parameters and conditions can be tested. Optimized conditions can then be scaled up to a bigger system. The preparative systems (pilot or industrial scale) are used to work with larger amount of samples and their configuration depends on the degree of automation. These systems are used to extract grams of compounds when working on pilot scale or kilograms on industrial scale. Furthermore, this equipment can contain one or more separators which collect the extract and depressurize the solvent. They are usually equipped with independent control of temperature and pressure which allows fractionating the extracted compounds by gradual depressurization. Different compounds are obtained within each separator, depending on their differential solubility in the supercritical fluid. If the separator is equipped with a refrigeration system, the most volatile compounds can be collected.

Carbon dioxide gas consumption can be minimized by implementing its regeneration system (Herrero et al., 2006).

To enhance the extraction yield and the selectivity of the targeted bioactive compounds, several additional techniques have been tested with SFE methodology. Higher yields can be obtained by cell wall hydrolysis preceded by biomass pretreatment with enzymes. Pretreatment facilitates solvent penetration through the biomass matrix. Ionic liquids can be designed to attain better dissolution of the targeted biomolecules and dissolve cell walls. Sudden supercritical CO_2 decompression in the material pretreatment promotes the rupture of the biomass cell walls which leads to higher solvent penetration. Ultrasound application can lead to similar results through the cavitation effect. It triggers the rupture of cell biomass which leads to increased solvent penetration and higher extraction yields. Despite all the combination techniques mentioned, there still is margin for further improvements. It is expected that a combination of different techniques can lead to higher yields and greater selectivity (Silva et al., 2016).

1.2.2. Extraction using enzymes

Enzyme-assisted extraction (EAE) is another promising technique for extracting bioactive compounds from plant material. This technique is usually used when phytochemicals are retained in the polysaccharide-lignin complex by hydrogen or hydrophilic bonding (Azmir et al., 2013). Enzymes can increase extraction efficiency, since they are able to catalyze reactions with high specificity and under mild conditions. These extractions are based on enzyme ability to degrade or disrupt cell walls and membranes. During this process, bioactive compounds are released and can be easily extracted (Pinelo et al., 2006; Puri et al., 2012). This extraction method can be successfully used as a pre-treatment step because it softens the structural integrity of the plant matrix (Sowbhagya & Chitra, 2010).

Various enzymes can be used for EAE (e.g. cellulase, α-amylase, pectinase and hemicellulase), depending on the material and the purpose of the extraction. These enzymes can be derived from different sources (e.g. bacteria, fungi, animal organs and plant extracts). When working with enzymes, it is crucial to understand their catalytic properties and optimal conditions at which the enzyme or enzyme combination can be used efficiently for extracting the material which is selected. EAE was successfully applied in phenolic compounds extraction from blackcurrant pomace using various enzymes (Landbo & Meyer, 2001). Another study observes that higher enzyme concentration significantly improves the recovery of phenolic antioxidants (Li et al., 2006).

Pretreating raw material with enzymes may result in decreased solvent consumption, the reduction of extraction time, increased yield and quality of the product (Meyer, 2010). However, there are several limitations regarding the cost of enzymes necessary to process large amounts of raw material. Furthermore, it might be difficult to scale up the process since enzymes can behave differently even with small changes in the process environment (e.g. oxygen level, temperature, nutrient availability). If these problems are solved, EAE can be a very promising technique for processing raw materials into valuable components. It can increase extraction

yields and enhance product quality since this technique enables the use of milder extraction conditions (Puri et al., 2012).

1.3. Extraction process optimization

In order to obtain the maximum benefit of the extraction process and establish the optimal conditions for best results, it is essential to improve the performance of the system (Araujo & Brereton, 1996). Traditionally, optimization has been carried out by monitoring the influence of one factor at a time while other parameters are kept constant. However, this optimization technique does not include the interactive effects among the variables studied. A large number of experiments need to be conducted to establish all necessary data, which leads to an increase of time spent one the experiment as well as consumption of reagents and materials.

The optimization of extraction procedures can be carried out by using multivariate statistic techniques and the most relevant is response surface methodology (RSM). It is a collection of mathematical and statistical techniques based on the fit of polynomial equation of experimental data. This technique is very useful when a response of interest is influenced by several variables. Using this methodology the levels of these variables can be optimized in order to achieve the best system performance.

When applying the RSM methodology, it is important to decide what kind of experimental design is going to be used. First-order model can be used when the data set is linear (Hanrahan & Lu, 2006). However, usually experimental data cannot be described by linear functions, thus experimental designs for quadratic response surface are more useful. There are several types of various designs, while the most popular are three-level factorial, Box-Behnken, central composite (CCD) and Doehlert designs (Bezerra et al., 2008).

RSM application consists of several steps and each of them is essential for research. Firstly, the independent variables which have major effects on the system have to be selected. Screening tests are useful for establishing which variables and their interactions present the most significant effects. When all independent variables are chosen, the experimental region should be defined. This step is highly dependent on the objective of the study and the experience of the researcher.

The simplest RSM model can be described with a linear function:

$$y = \beta_0 \sum_{i=1}^{k} \beta_i x_i + \varepsilon; \quad (1)$$

where k is the number of variables, β_0 is the constant term, β_i represents the coefficients of the linear parameters, x_i represents the variables and ε is the residue of the experiments.

When the results and the interactions of independent variables are more complicated, a second-order model is used to evaluate curvature. This model is used when additional, such as second-order effects are significant in the experiment. This polynomial model should contain additional terms which describe the interactions between different experimental variables. A model for a second-order interactions is presented in the following equation:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \; ; \quad (2)$$

where β_{ij} represents the coefficients of the interaction parameters.

During various extractions, the main objective is to find the critical point (maximum, minimum or saddle). For this purpose, the polynomial function has to be used. It contains quadratic terms expressed with the equation presented below:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \; ; \quad (3)$$

where β_{ii} represents the coefficients of the quadratic parameter.

To calculate the parameters in the third equation, all studied variables have to be analyzed on at least three factorial levels using symmetrical response surface design. All of the better known models have been mentioned before and they differ from one another with respect to the selection of experimental points, the number of levels for variables, and the number of runs and blocks (Bezerra et al., 2008).

The central composite design consists of a full factorial or fractional factorial design, an additional design and a central point (**Fig. 1.5**). The star design is often used as an additional design and it contains experimental points which are at specific distance (α) from the center. This design was introduced by Box and Wilson (Box & Wilson, 1951).

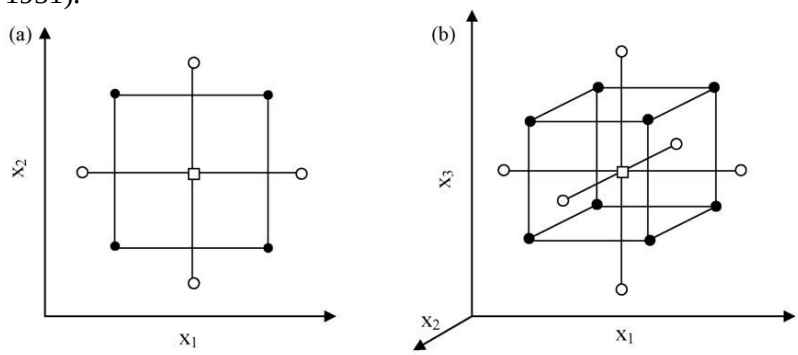


Figure 1.5. Central composite designs for the optimization of two (a) and three (b) variables. (●) are points of factorial design, (○) are axial points and (□) is the central point (Bezerra et al., 2008)

All factors in this design are studied at five levels (-α, -1, 0, +1, +α) and it requires a specific experiment number:

$$N = k^2 + 2k + c_p; \quad (4)$$

where k is the factor number and c_p is the replicate number in the central point.

The specific distance of additional experimental points depends on the number of variables and can be calculated with a formula:

$$\alpha = 2^{(k-p)/4}. \quad (5)$$

There are two main varieties of central composite design: face centered and rotatable. In face centered CCD with three factors, (**Fig. 1.5**) the factorial points are

in the corners of the cube representing the experimental domain, the axial points are in the center of each face of the cube and the center points are the replicates in the center of the cube. These replicates are necessary to estimate the variability of the experimental measurement and their number may vary from three to six. For face centered CCD, the chosen value of α is 1. The most widely used experimental design is rotatable CCD. The design is called rotatable, if the precision of the estimated response surface at any point depends only on the distance and not on the direction of the point from the center point of the design (Sahoo, Barman, & Davim, 2011). These designs do not favor one direction over another when the response surface is explored. The additional points can be designated as the second block and they provide a link between the experiment design blocks. The star points improve the estimation of second-order effects needed to characterize curvature (Anderson & Whitcomb, 2016).

Central composite design has been applied in many analytical procedures and processes because it is a powerful tool for screening and optimization. It can help us optimize the extraction of bioactive compounds in order to achieve higher yields or optimal values of antitumor, antimicrobial, antibacterial, antiviral and anti-inflammatory activity.

1.4. Nutrition and antioxidants

Oxygen is one of the most important elements in nature and it is essential for life. However, it can play a contradictory role and act as a toxic substance. This important element is required by prokaryotic and eukaryotic cells for energy production via electron transport chain in the mitochondria. Mostly it is consumed in the form of diatomic molecule since this configuration exists in our atmosphere. On the other hand, this element is toxic to anaerobic bacteria and forces them to develop a variety of mechanisms to cope with its increasing concentration. Oxygen concentration in the atmosphere is a dynamic parameter which constantly changes. Even small changes in the concentration of oxygen may lead to changes in the biochemical response of a living cell (Halliwell & Gutteridge, 2007; Kohen & Nyska, 2002).

Chemically, every compound that can accept electrons is an oxidant or an oxidizing agent, while a substance that donates electrons is a reductant or a reducing agent (Prior & Cao, 1999). Oxidation is a process of losing an electron; an oxidant accepts electrons, while causing another substance to become oxidized. The oxidation process (a gain of oxygen) is always accompanied by a reduction process (usually a loss of oxygen) (Hrbac & Kohen, 2000). These are the so-called redox reactions and they are very important for numerous biochemical pathways, biosynthesis and regulation (Shapiro, 1972). These mechanisms are crucial for understanding biological oxidation and the radical effect of an antioxidant. In biological systems, reductants and oxidants are called antioxidants and pro-oxidants (Hrbac & Kohen, 2000).

Oxygen-derived pro-oxidants can damage lipids, DNA, protein or even the cell's defense systems. In general, these pro-oxidants are referred to as reactive oxygen species (ROS). Furthermore, free radicals can originate from nitrogen

(reactive nitrogen species (RNS) or sulfur (reactive sulfur species (RSS). RSS can easily arise from ROS reaction with thiols (Carocho & Ferreira, 2013). Pro-oxidants can be classified into two groups: radicals and non-radicals. The radical group contains compounds such as nitric oxide radical ($NO^{\bullet}$), superoxide ion radical ($O^{\bullet}_2^-$), hydroxyl radical ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$), and one form of singlet oxygen ($^1O_2$) (Jurgen, Maurizio, & Lester, 2003). These radicals contain at least one unpaired electron in the shells around their atomic nucleus and they can exist independently. All these compounds are highly reactive until they gain stability by donating or accepting an electron (Barry Halliwell & Gutteridge, 1999). The non-radical group contains a large variety of substances, some of which are extremely reactive. Living cells can produce high concentrations of hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), organic peroxides, aldehydes, ozone (O_3) and singlet O_2. Previous literature marks radicals with a superscripted dot ($R^{\bullet}$); this helps to distinguish them from the other reactive oxygen metabolites. The source of ROS and RNS can be both exogenous and endogenous (**Fig. 1.6**) (Kohen & Nyska, 2002).

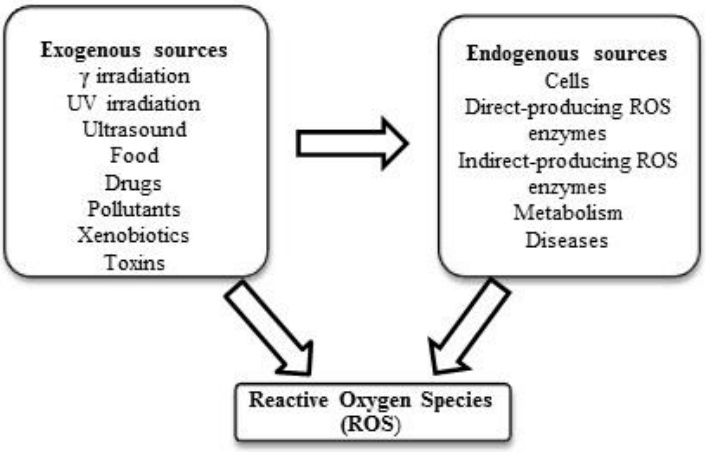


Figure 1.6. Sources of reactive oxygen species (Kohen & Nyska, 2002)

1.4.1. The mechanism of lipid oxidation

There are three major pathways that are responsible for lipid oxidation: auto-oxidation, photo-oxidation and enzymatic oxidation (Carlsen, Møller, & Skibsted, 2005).

Auto-oxidation is a free radical chain reaction which consists of three main stages: initiation, chain propagation and chain termination (**Fig. 1.7**) (Frankel, 2005).

Initiation:	$LH + X^{\bullet} \rightarrow L^{\bullet} + HX$
Chain propagation:	$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ $LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH$ $LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH$ $LOOH + LO^{\bullet} \rightarrow LOO^{\bullet} + LOH$ $LO^{\bullet} + LH \rightarrow L^{\bullet} + LOH$ ${}^{\bullet}OH + LH \rightarrow L^{\bullet} + H_2O$
Chain termination:	$L^{\bullet} + L^{\bullet} \rightarrow L\text{-}L$ $L^{\bullet} + LOO^{\bullet} \rightarrow LOOL$ $LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$
Chain termination with antioxidant:	$QH_2 + LOO^{\bullet} \rightarrow QH^{\bullet} + LOOH$ $QH^{\bullet} + LOO^{\bullet} \rightarrow Q^{\bullet} + LOOH$

Figure 1.7. The peroxidation of LH initiated by X radical and inhibited by antioxidant QH_2

All primary reactions where active free radicals are formed may be assigned to the initiation stage. In the presence of initiators (light, trace transition metals, free radicals) unsaturated fatty acids (LH) lose a hydrogen atom and form an alkyl radical $L^{\bullet}$. When oxygen concentration is high enough, it can react with lipids to form the $LOO^{\bullet}$ radical. The new radical reacts with another LH to form hydroperoxide (LOOH), a primary oxidation product. Hydroperoxide or LOOH, a principle product of lipid peroxidation, can also be a source of free radicals when destructed. The destruction of LOOH compound and formation of an active free radical is classified as a special stage called degenerate chain-branching. The $L^{\bullet}$ radical is very active and is rapidly converted into $LOO^{\bullet}$ due to the extremely fast reaction with oxygen: $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$. As a result, this is the only principle chain-carrying free radical. This chain reaction can be suspended by scavenging the chain-carrying radical $LOO^{\bullet}$ which requires a donor of H-atom, usually an antioxidant. Primary OH-substituted phenoxy radical $QH^{\bullet}$ is converted into semiquinone $Q^{\bullet-}$ due to fast deprotonation ($QH^{\bullet} \rightarrow Q^{\bullet-} + H^{+}$). These reactions of polyphenols occur in aqueous phase at neutral pH, while the phenoxy radical produced from monophenolics exists even in the aqueous medium as a non-charged specie (**Fig. 1.8**) (Roginsky & Lissi, 2005).

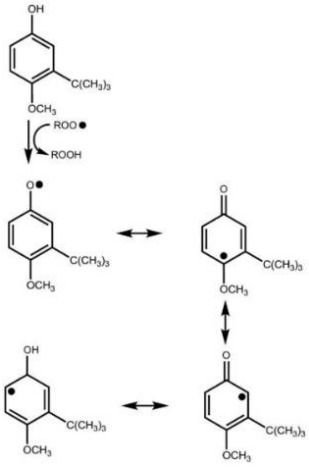


Figure 1.8. Resonance stabilization of a free radical by a phenolic (Decker et al., 2010)

Photo-oxidation. When oxygen reaches the singlet state ($^1O_2$), light energy promoted by pigments that act as sensitizers induces photo-oxidation. Oxygen in this new condition is highly electrophilic and can react rapidly with unsaturated lipids. The mechanism of this reaction is slightly different from free-radical auto-oxidation. In the presence of sensitizers, the double bond of fatty acid (FA) residue interacts directly with singlet oxygen. Moreover, the photo-oxidation reaction is much faster than auto-oxidation; it occurs more than 1,500 times faster than the reaction between triplet oxygen and a polyunsaturated FA (Bartosz, 2014).

Enzymatic oxidation is induced by lipoxygenases which are common in plants, animals and microorganisms. These enzymes belong to the non-heme iron-containing fatty acid dioxygenases family. It differs from the free-radical reaction with respect to the formation of hydroperoxides in a defined position of fatty acids chains. Lypoxygenases use molecular oxygen to catalyze the stereospecific and regiospecific oxygenation of polyunsaturated fatty acids with 1-*cis*,4-*cis*-pentadiene moieties. Furthermore, the new fatty acid peroxy free radical separates hydrogen from other unsaturated fatty acid molecules. The newly formed hydroperoxy dienes are responsible for the off-flavor and lipid oxidation in different food products. Sometimes these reactions may be desirable but they have to proceed at a low degree. The oxidation products of lipoxygenases are responsible for typical flavor of cucumbers, fresh fish and virgin olive oil. Cyclooxygenase enzymes can also catalyze the addition of molecular oxygen to polyunsaturated fatty acids. As a result, these compounds are converted into biologically active endoperoxides, intermediates in the formation of fatty acids to prostaglandins (Bartosz, 2014).

Lipid peroxidation can also be initiated by metal ions. These metal ions can be found naturally in food components and gained from the environment or metal equipment. It is believed that transition metal ions are the main source of the hydroxyl radical in biological systems. Furthermore, transition metals accelerate the decomposition of lipid hydroperoxides (Bartosz, 2014).

1.4.2. Antioxidants

The beneficial influence of many foodstuffs and beverages on human health has been recognized to originate from the chain-breaking antioxidant activity (AOA) of natural food components. An antioxidant can be any substance that, when present at low concentration compared to that of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell, 1995). When the increased formation of ROS overwhelms the body's antioxidant protection it can result in DNA damage, lipid peroxidation, protein modification and other harmful effects. These changes can cause numerous diseases, including cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders (e.g. Alzheimer's disease, Parkinson's disease), chronic inflammation and many others (Jomova & Valko, 2011). Various antioxidants from dietary sources can terminate ROS attacks thus helping to prevent diseases and health problems. It is important to look for new, safe and effective antioxidants from natural sources. Many foodstuffs and beverages have beneficial health effects on our organisms and it has been recently recognized to originate from their antioxidant activity (Charles, 2013).

Natural antioxidants in foods can be of different origins; they can occur as endogenous compounds in one or more food components or form in reactions during processing. They can also be added as food additives isolated from natural sources. Many natural antioxidants come from the plant kingdom and their activity can range from very slight to extremely high. All these natural compounds are the defense system in vivo and they act in several ways. They inhibit the formation of ROS and free radicals by sequestering metal ions, reducing hydroperoxides and hydrogen peroxide, and quenching superoxide and singlet oxygen. Phenolics are the main antioxidant components in food products, while in plant oils and fats monophenolics (tocopherols) are dominant. Vegetables, tea, coffee and wine typically contain water-soluble polyphenols. Many natural phenolics and flavonoids (flavonols, isoflavones, flavones, catechins and flavanones) belong to the group of the most potent H-donors (Roginsky & Lissi, 2005).

Polyphenolic compounds vary in structure and can have a single-ring structure (hydroxybenzoic acid, hydroxycinnamic acid), while flavonoids can be found in dimers, trimers and polymers (**Fig. 1.9**). Flavonoids can be further classified into anthocyanins, flavan-3-ols, flavones, flavanones and flavonols. Flavonoids generally consist of two benzene rings linked by an oxygen-containing heterocycle. Dietary polyphenolics have higher antioxidant activity as compared to that of the essential vitamins (Tsao & Deng, 2004).

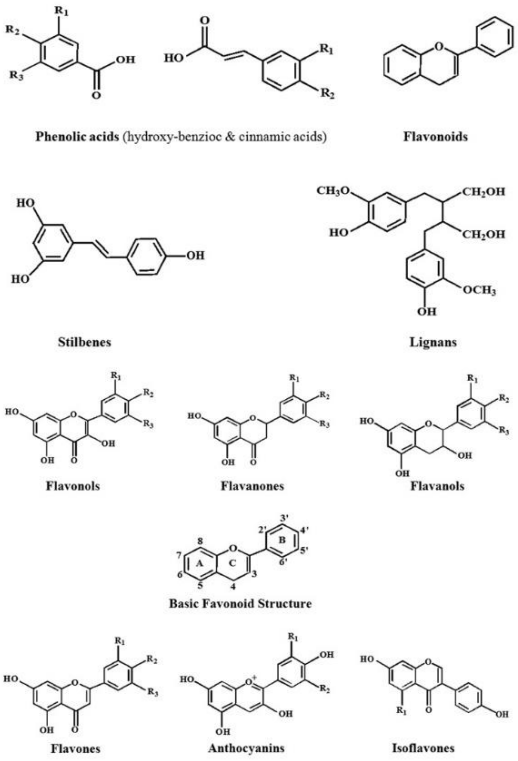


Figure 1.9. Chemical structures of different classes of polyphenols and sub-classes of flavonoids in plants (Pandey & Rizvi, 2009).

1.4.3. Methods for determining antioxidant activity

Antioxidant activity is an important characteristic of dietary components. It can be determined by the antioxidant composition and antioxidant properties of the constituents. Antioxidant activity is considered as a significant parameter which determines dietary value of many different food products. The effectiveness of antioxidants can be influenced by a number of different factors, such as their structural features, concentration, temperature, type of oxidation substrate and physical state of the system. The reaction kinetics is also an important parameter because it involves the rate at which the antioxidant reacts, the thermodynamics of the reaction, and the extent to which the antioxidant reacts (Antolovich et al., 2002). All these factors have to be considered carefully to select a suitable antioxidant for specific application. Antioxidant activity measurement tools and equipment have advanced remarkably during the last few decades. Nowadays antioxidant activity evaluation assays have become much faster and more sensitive; automated detection technologies have been applied in the measurements. An antioxidant activity assay should be simple, reproducible and adaptable for hydrophilic and lipophilic antioxidants. Furthermore, it has to measure the chemistry occurring in potential application, use a method with a defined endpoint and chemical mechanism, and

utilize a biologically relevant radical source (Prior, Wu, & Schaich, 2005; Shahidi & Zhong, 2015).

Antioxidants can disable radicals with two major mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET). Both reactions can stop lipid peroxidation, but the kinetics and potential side reactions of the two mechanisms are different. The dominating mechanism can be determined by the antioxidant structure and specific properties, solubility and partition coefficient and system solvent. Therefore, it is suggested to apply protocol where more than one property can be measured since polyphenols have multiple activities and the dominant activity depends on the medium of the substrate of testing. HAT-based methods measure the ability of an antioxidant to quench free radicals using hydrogen atom donation, which is believed to be the principle of antioxidant activity. These reactions are rather fast, typically completed in a few seconds or minutes. The SET method detects the ability of an antioxidant to transfer electrons to reduce any compound (radical, carbonyl or metal). In general, both antioxidant mechanisms occur together but their balance can be determined by the structure and pH value of the antioxidant. Electron transfer reactions are slow and they can require a long time to complete.

Several methods are necessary to measure the total antioxidant activity capacity. In order to get the best results, both lipophilic and hydrophilic capacities have to be measured and both hydrogen atom transfer (radical quenching mechanism) and electron transfer (radical reduction) have to be analyzed. It is suggested to use three main assays: oxygen radical absorbance capacity (ORAC) assay, Folin-Ciocalteu method and the Trolox equivalent antioxidant capacity (TEAC) assay (Huang, Boxin, & Prior, 2005).

ORAC method measures antioxidant inhibition of peroxyl radical chain breaking the antioxidant activity by hydrogen atom transfer (Ou, Hampsch-Woodill, & Prior, 2001). The peroxyl radical reacts with a fluorescent probe (fluorescein) and forms a non-fluorescent product; the changes can be easily measured using fluorescence. Antioxidant capacity is determined by a decreasing rate of fluorescence and amount of the product. The calculation of the protective effects of antioxidants accounts for the delay, initial rate and total extent of inhibition in a single value. The results are reported as Trolox equivalents. $ORAC_{FL}$ assay is limited because it measures only hydrophilic chain breaking activity, although lipophilic antioxidants are particularly important in lipid oxidation. Consequently, L-ORAC assay has been adapted to measure lipophilic antioxidants using a solution of 50 % acetone and 50 % water (v/v) containing 7 % of randomly methylated β-cyclodextrin (RMCD) to solubilize the antioxidants (Huang et al, 2002; Prior et al., 2003).

$$ROO^{\bullet} + \text{probe (fluorascent)} \rightarrow ROOH + \text{oxidized probe (loss of fluorescence)}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

$$ROO^{\bullet} + A^{\bullet} \xrightarrow{\text{fast}} ROOA$$

This reaction is temperature-sensitive thus close temperature control is essential. It is recommended to use multichannel liquid handling system, coupled

with a fluorescence microplate reader, preferable with 48-well format (Prior et al., 2003).

Folin-Ciocalteu (FC) assay is used to measure the total content of phenolic compounds. Testing systems consist of tungstate and molybdate in highly basic medium of aqueous Na_2CO_3 (5−10 %). Phenolic compounds are oxidized in a basic medium which results in the formation of superoxide ion radical. This radical reacts immediately with molybdate and forms molybdenium oxide. This newly formed element has a very intensive absorbance, near 750 nm (Folin & Ciocalteu, 1927).

$$Na_2WO_4/Na_2MoO_4 \rightarrow (phenol - MoW_{11}O_{40})^{-4}$$
$$Mo(VI)(yellow) + e^- \rightarrow Mo(V)(blue)$$

This method is simple, sensitive and precise. However, the reaction is slow at acid pH and not precise enough thus it has been improved with a molybdotungstophosphoric heteropolyanion reagent (Singleton, Rossi, & Rossi, 1965; Singleton, Orthofer, & Lamuela-Raventes, 1998).

$$3H_2O - P_2O_5 - 13WO_3 - 5MoO_3 - 10H_2O$$
$$3H_2O - P_2O_5 - 14WO_3 - 4MoO_3 - 10H_2O$$

This reagent reduces phenols more specifically and maximum absorbance is reached at 765 nm. Phenolic compounds obtained by this method are most frequently expressed in gallic acid equivalents. This method is not specific, as it detects all phenolic groups found in extracts, including those in the extractable proteins. Furthermore, the reducing substances (i.e. ascorbic acid) can affect the results (Roginsky & Lissi, 2005).

$ABTS^{\bullet+}$ assay. TEAC assay is based on the scavenging ability of antioxidants to the long-life radical anion $ABTS^{\bullet+}$ (Miller et al., 1993). $ABTS^{\bullet+}$ is oxidized to its radical cation which is intensely colored. When an antioxidant reacts with $ABTS^{\bullet+}$ (**Fig. 1.10**), it is converted into non-colored form and these changes can be measured spectrophotometrically.

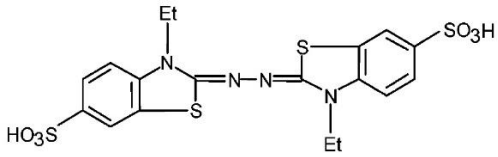


Figure 1.10. The chemical structure of the $ABTS^{\bullet+}$ radical (2,2′-azinobis(3-ethylbenzothiaziline-6-sulfonate)

The $ABTS^{\bullet+}$ radical can be used to measure both hydrophilic and lipophilic antioxidant capacities because it is soluble in both aqueous and organic solvents and can be used over a wide pH range. This radical is formed is either a chemical reaction or enzyme reactions. The former requires a long time or high temperature, while the latter is faster and the reaction conditions are milder (Prior, Wu, & Schaich, 2005).

$DPPH^{\bullet}$ method is one of the oldest methods to measure antioxidant capacity. Basically, it is a stable organic nitrogen radical of a deep purple color (**Fig. 1.11**).

Unlike $ABTS^{\bullet+}$ radical, it is commercially available, does not have to be generated before measurements and is likely more selective in the reaction with H-donors. The antioxidant activity assay is based on measuring the loss of $DPPH^{\bullet}$ color at 515 nm after reaction with the analyzed material (Brand-Williams, Cuvelier, & Berset, 1995). The color can be affected in either radical reaction with hydrogen or, most usually, in reduction by single electron transfer. This method is simple, easy and rapid which makes it rather popular for antioxidant screening. It is difficult to test the compounds that have spectra that overlap $DPPH^{\bullet}$ at 515 nm. Many antioxidants which react quickly with peroxyl radicals may react slowly or even be inert to $DPPH^{\bullet}$. The reason for this is that a stable nitrogen radical is not similar to the highly reactive and transient peroxyl radicals (Prior et al., 2005).

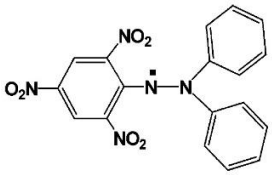


Figure 1.11. The chemical structure of the $DPPH^{\bullet}$ radical (2,2-diphenyl-1-picrylhydrazyl)

Frap assay (ferric reducing antioxidant power) is based on the capability of phenolic antioxidants to reduce Fe^{+3} to Fe^{+2} (Benzie & Strain, 1996; Pulido, Bravo, & Saura-Calixto, 2000). The reduction is accompanied by the formation of a colored compound with Fe^{+2} when 2,4,6-trypryridyl-*s*-triazine (**Fig. 1.12**) (TPTZ) is present. Fe^{+2}-TPTZ is of an intensive blue color and it can be monitored spectrophotometrically at 593 nm. This method is inexpensive, the reagents are simple to prepare and the results are highly reproducible.

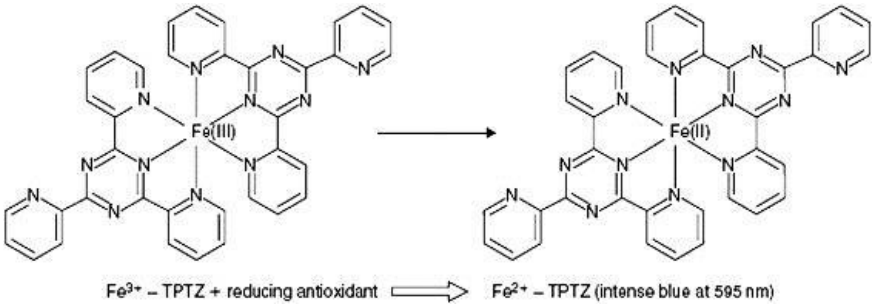


Figure 1.12. The reduction reaction of FRAP assay

QUENCHER procedure. Individual antioxidant compounds might be present in different forms, some of which can be completely soluble in a solvent, while others might be completely insoluble. The QUENCHER procedure is suggested to overcome this problem. This method is based on the direct analysis of solid samples by mixing them with free radicals or other substances for antioxidant activity measurements and analysis using spectrophotometric methods. This method is quick, easy, new, cheap and reproducible; the name for the procedure, QUENCHER, originates as an acronym of this description. (Gökmen, Serpen, & Fogliano, 2009). It can be used with various antioxidant activity assays.

1.5. The characterization of selected cereal and berry processing by-products

1.5.1. Cereal grains

Different cereals and cereal products are widely spread around the world. It is the primary source of carbohydrates for humans and animals. Wheat and rye grains are the two main cereals used for bread making. According to FAO, the annual cereal production in 2014 exceeded 2,800 million tons, with three major species (maize, wheat and rice). Wheat production during the past few years is above 700 million tons and rye cereal production is lower, about 15 million tons (FAO, 2014).

When working with cereals, it is crucial to understand the structure of industrially important cereal grain. All the members of the grass family *Gramineae*, including cereal grains, produce dry one-seeded fruit which should be called caryopsis instead of "kernel" or "grain". A wheat caryopsis consists of an inner side (seed) and the outer part (seed coat or pericarp). A seed can be divided into smaller parts: an embryo (germ), an endosperm, a nucellar epidermis and a seed coat.

All cereal grains consist of the same parts and their relationship to each other is very similar. Grains are comparatively stable during storage because their chemical constituents are often separated from each other by cell walls or other barriers, and they have relatively low water activity. Furthermore, grains often contain both degrading enzymes and the substrates of these enzymes. The degradation processes can easily start if these constituents come into contact and a proper water activity threshold is passed. The system is stable if the substrate and the enzyme are kept away from reaching each other (Shewry, 2010).

1.5.1.1. Wheat bran

Wheat is the most popular grain all over the world. It can easily grow under various environmental conditions. Wheat grain can be divided into two groups by the force which is needed to crush their kernels (soft and hard). Hard wheat is mainly used for pasta production, while soft grain is popular for producing other products (bread, cookies and biscuits). The longitudinal and cross sections of a wheat kernel are presented in **Figure 1.13**. The size and shape of a wheat kernel can vary depending on their genus or specie. Moreover, the wheat variety and agro-climatic conditions are important factors (Dziki & Laskowski, 2005). The European wheat grain weighs about 55 mg. On the dorsal side, they are rounded, and on the ventral side, which runs through the entire length of the kernel, they have a crease. If the two formed cheeks mask the depth of the crease, it becomes difficult to separate the bran from the endosperm and to achieve good grain fractionation. Furthermore, the crease can become a good hiding place for harmful microorganisms and dust.

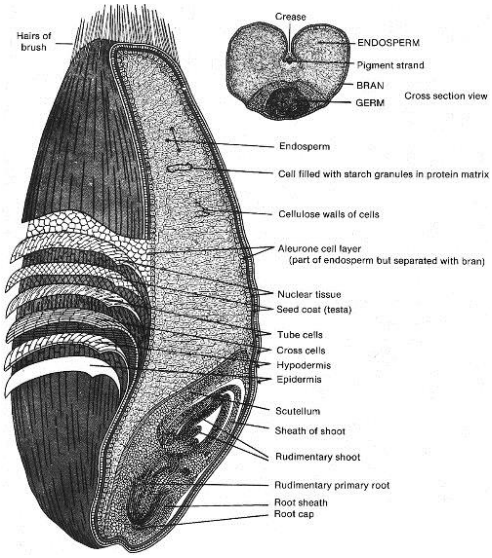


Figure 1.13. Longitudinal and cross sections of a wheat kernel (Bass, 1988)

The entire seed is surrounded by the pericarp which is composed of several layers. It consists of about 6 % protein, 2 % ash, 20 % cellulose and 0.5 % fat with the remainder being non-starch polysaccharide. The kernel is surrounded by one-cell-thick aleurone layer which completely surrounds the kernel and covers the starchy endosperm of the germ. This layer along with the nucellar epidermis, the seed coat and the pericarp is removed during milling and forms the so-called "bran" fraction. Aleurone cells cover the endosperm and contain a large nucleus and a large number of aleurone granules. Their complex structure is presented in **Figure 1.14**. This outer layer is relatively high in enzyme activity, ash, protein, total phosphorus and lipid content (Shewry, 2010).

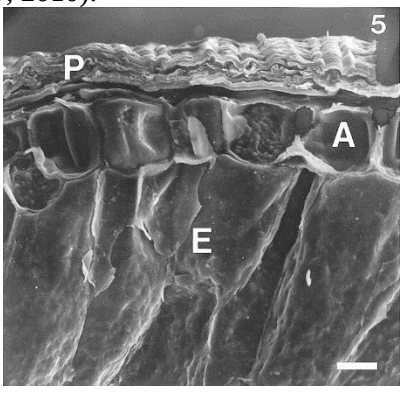


Figure 1.14. A scanning electron micrograph of a cross section of a hard winter wheat kernel (Pericarp (P), aleurone layer (A), and endosperm (E). Bar is 20 μm (Hoseney & Seib, 1974)

During wheat flour milling, flour extraction rate ranges from 73 to 77 % depending on what milling process or cereal variety is used (Elliott et al., 2002). Other parts of cereal grain go to the by-product milling stream (23−27 %) which

consists of wheat germ and some parts of the endosperm. Bran fraction also includes the aleurone layer, because it is impossible to separate all layers from each other (Campbell et al., 2006).

Cereal bran is usually used as animal feed and just a minor part is sold as commercial bran for food purposes (Prückler et al., 2014). Since it is important to use earth resources more efficiently, new value-added applications for wheat bran need to be examined. During the last decade, wheat bran usage in feeding and food industry has increased. More and more products with added cereal bran are available in supermarkets. Wheat bran can be added to different products and its percentage in the product may vary widely, depending on the products to which they are added. Baked goods and cereal products are the most common products with this additive but it is also used in dairy products, sauces, soft drinks and soups.

Bran is a rich source of various bioactive components, such as dietary fibers, essential fatty acids, phytosterols, antioxidants, minerals, vitamins and proteins. Bran is the "nutritional storehouse" of grains. Cereal bran can contain various phenolic compounds presented in **Figure 1.15**. Previous experiments have shown that bran is a key factor in determining the health benefits of whole grain products. It is proven that bran fraction can modulate consumers' sense of hunger and satiety, influence the glycemic, lipid and inflammatory status. A major part of research focuses on natural antioxidants which may act as free radical scavengers, reducing agents and quenchers of the singlet oxygen because the oxidation of cell components is often involved in disease-causing mechanisms (Prückler et al., 2014).

All these different valuable compounds can be divided into four major groups:

- soluble and insoluble dietary fiber (arabinoxylan and β-glucan);
- sugars and their derivatives (starch, glucose, succinic acid);
- secondary plant metabolites (ferulic acid, phytic acid);
- proteins for specific amino acids production (Apprich et al., 2014).

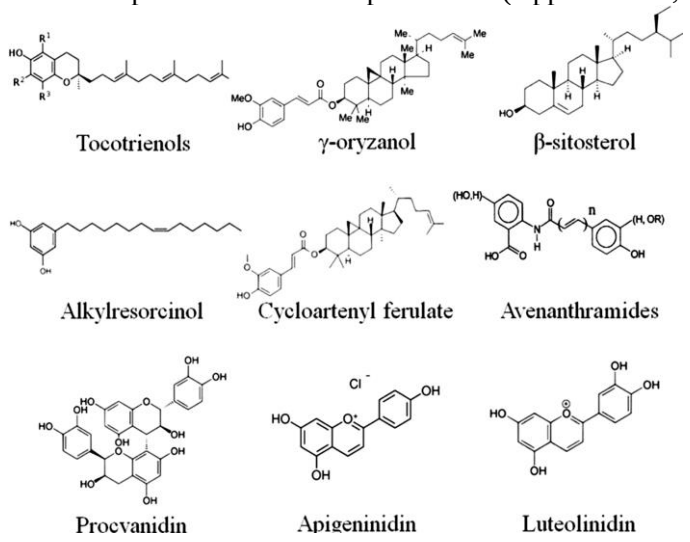


Figure 1.15. The structures of major phytochemicals extracted from various cereal brans (Patel, 2012)

Starch is one of the major compounds in wheat bran (accounting for 14−25 %, depending on the degree of milling) and it consists of two different types of granules (A-type and B-type). These granules differ in diameter and chemical and functional properties. A-type granules are larger (>10 μm) and make up about 70 % of commercial wheat starch, while B-type granules are smaller than 10 μm and comprise about 30 % of commercial wheat starch. Wheat bran starch is different from the commercial wheat starch in that it has different functional and chemical properties (Xie, Cui, Li, & Tsao, 2008). Starch obtained from wheat bran can be hydrolyzed to glucose and then used for various purposes, for instance, as a sugar feedstock for fermentation to produce lactic acid, succinic acid, ethanol or butanol. Lactic acid is widely used in the food, cosmetic, pharmaceutical and chemical industries mainly as a preservative or flavor component. Succinic acid is used to control acidity or as a preservative and flavoring agent in food. Furthermore, it is a precursor for other important compounds such as 1,4-butanediol, tetrahydrofuran, γ-butyrolactone and adipic acid (Apprich et al., 2014).

Wheat bran contains cellulose, cuticle materials and complex xylans which with high arabinose-to-xylose ratio (A/X) and contain ferulic acid dehydromers as cross-linkers between polymer chains (Hemery et al., 2010). The pericarp and testa layers are rich in lignin, and testa contains almost all of the alkylresorcinols of the grain. These compounds act as antioxidants and exert the anti-mutagenic and anti-bacterial activity; they are commonly used as biomarkers in human body to measure whole-grain intake in the diet (Prückler et al., 2014).

The alleurone layer cell walls are composed of relatively linear arabinoxylans with low arabinose-to-xylose ratio (Saulnier et al., 2007). Arabinoxylan is based on a linear β-D-(1→4)-linked xylopyranose backbone which can be substituted by C(O)-2, C(O)-3 or both in irregular patterns (Brillouet & Joseleau, 1987). The alleurone layer also contains wheat β-D-glucans which are water soluble, produce different beneficial health effects and can be used as an ingredient in functional food production (Prückler et al., 2014).

The European Food Safety Authority (EFSA) has applied several claims regarding wheat bran consumption and its positive impact on human health:

- dietary fiber content of wheat bran contributes to an increase of facial bulk (health claim ID3066);
- it reduces bowel transition time (health claim ID839,3067,4699);
- it stabilizes the blood-glucose level after a meal (health claim ID830).

In order to achieve these physiological effects, people have to consume at least 25 g of dietary fiber from wheat bran. Furthermore, to control blood-glucose level people have to ingest at least 8 g of arabinoxylan-rich fiber produced from wheat endosperm consisting of at least 60 % arabinoxylan per 100 g of available carbohydrates (European Food Safety Authority, 2010).

Dietary fiber is an edible part of plants or analogue carbohydrates that are resistant to digestion and absorption through the small bowel. It can be partially or fully fermented in the large bowel, yielding short chain fatty acids, hydrogen, carbon dioxide and methane as fermentation products (Escudero & Gonzalez, 2006). Vitaglione (2008) (Vitaglione, Napolitano, & Fogliano, 2008) has suggested that

phenolic compounds may be released from soluble dietary fiber by esterase and absorbed to various extents through the intestine. When these compounds get into the bloodstream, the whole body is positively affected. Microbiota in the lower gut helps to absorb phenolic compounds from the dietary fiber. Ferulic acid is found randomly esterified with a single α-L-arabinofuranoze residue in C-5. Arabinoxylan can possess anti-oxidant properties and is the dominant phenolic compound in wheat; 98 % of total ferulic acid is located in the bran layer (Lempereur, Rouau, & Abecassis, 1997).

Bran layer has a very important physiological function; it protects the seed from the environment. As a result, this layer is a rich source of bioactive components, mainly phenolic, that can be extracted and used to enrich our diet. The major part of phenolic compounds found in wheat bran consists of phenols containing one aromatic ring (ferulic acid, sinapic acid, *p*-coumaric acid). Ferulic acid (3-(4-hydroxy-3-methoxyphenyl) propionic acid) is a bioactive compound that possesses anti-oxidant, anti-microbial, anti-inflammatory, anti-thrombosis and anti-carcinogenic activity (Ou & Kwok, 2004). Besides, this acid can be used as raw material for the production of natural vanillin. Generally, ferulic acid is extracted from the lignin-phenolic-carbohydrate complex using alkaline or acid hydrolysis at elevated temperatures. Subcritical water extraction can be a very promising technique because no toxic, flammable or expensive organic solvents have to be used (Pourali, Asghari, & Yoshida, 2010).

Wheat bran can be used as a source of protein (13−18 %). It has a different chemical structure as compared to wheat flour; the endosperm proteins are composed of glutenins and gliadins, while the bran proteins primarily contain albumins and globulins. Furthermore, bran fraction contains higher amount of lysine, arginine, alanine, asparagine and glycine, and less glutamine, proline, phenylalanine and Sulphur-containing amino acids. As a result, its biological and nutritional value is higher (Apprich et al., 2014; Di Lena, Vivanti, & Quaglia, 1997).

Wheat bran contains about 3.5−3.9 % of oil; however, not much research has been done regarding this fraction. The most abundant fatty acid in wheat germ and bran oil is linoleic acid (~57 %), followed by oleic acid (~15 %) and palmitic acid (22 %). This oil also contains a relatively high content of carotenoids (39 μg/g wheat bran oil) (El-Shami et al., 2011; Jung et al., 2010). Due to the amphiphilic nature of these resorcinolic lipids, the extraction yield is higher using polar organic solvents than supercritical carbon dioxide (Rebolled et al., 2013). Oil obtained by SC-CO_2 is of better quality as compared to hexane-extracted oil, due to lower acid and peroxide value. Furthermore, this oil had longer oxidation induction period during rancimat test (Jung, Kang, & Chun, 2012).

1.5.1.2. Rye bran

The rye (*Secale cereale L.*) kernels are very similar to wheat. Their caryopsis is about 6−8 mm in length and 2−3 mm in width. These cereals have a similar grain structure with a ventral crease. Rye caryopsis consists of a pericarp, a seed coat, a nucellar epidermis, a germ and an endosperm. Like in wheat grain, the endosperm is surrounded by a single layer of aleurone cells (**Fig. 1.16**). The endosperm cell starch

is embedded in a protein matrix. Rye starch consists of large lenticular and small spherical granules (Shewry, 2010).

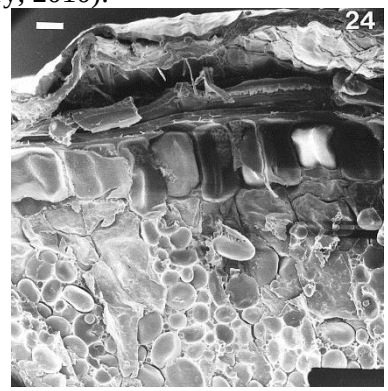


Figure 1.16. A scanning electron micrograph of the outer part of a rye kernel. Bar is 20 μm (Shewry, 2010)

In comparison to wheat bran, there is not so much research investigating rye bran. Rye flour is not as popular as wheat flour around the world, although rye is the most important source of dietary fiber in the Nordic countries. Rye bran composition closely resembles that of wheat; it contains similar compounds, even though the quantity or proportions of these components may differ extensively. For example, the levels of dietary fiber are similar but the relative proportions of fiber components (arabinoxylan, β-glucan, cellulose, fructan and lignin) are different. Rye bran contains less cellulose but more β-glucan and fructan than wheat bran. The quantities of such bioactive components as tocopherols, tocotrienols, total folate, sterols/stanols, phenolic acids and lignans do not differ to a large extent. Rye bran contains more alkylresorcinols but less glycine betaine than wheat (Kamal-Eldin et al., 2009).

Due to differences in grain type, varietal heterogeneity and processing, the analysis of starch content in rye bran might produce a wide variety of results from different samples. The starch content in rye bran is similar to wheat bran; it can range from 16 to 28 g/100 g DW. Similar results were observed in studies using a roller mill; the starch content was ~30 g/100 g DW (Kamal-Eldin et al., 2009; Katina et al., 2007). Furthermore, the milling process has influence over the ash content, which is used as an indicator of bran content in the milling industry, since all the minerals are concentrated in this fraction (Glitsø & Bach Knudsen, 1999).

Dietary fiber is the predominant constituent of rye bran (41−48 g/100 g). Its quantity depends on how efficiently the endosperm can be separated from the outer part of the seed. The main component of dietary fiber is arabinoxylan (21−25 g/100 g), followed by fructan (6.6−7.2 g/100 g) and β-glucan (4.3−5.3 g/100 g). Rye bran has been revealed to contain nearly twice as much of fructan and β-glucan than wheat bran; it also incorporates relatively high levels of cellulose (5.0−6.0 g/100 g) and Klason lignin (3.5−4.4 g/100 g). Rye and wheat bran contain comparable amounts of arabinoxylan and present similar arabinose-to-xylose ratio (A/X ~0.5).

Rye bran presents double the amount of β-glucan and fructan but half of the volume of cellulose as compared to wheat bran (Kamal-Eldin et al., 2009).

Tocols are mainly accumulated in the bran of rye and wheat, while tocopherols are concentrated in the germ fraction (Ko et al., 2003). α-tocotrienol levels are higher than β-tocotrienol levels in rye bran, but wheat bran presents the opposite dependency. Usually, low levels of tocotrienol can be due to higher starch content in the material (Kamal-Eldin et al., 2009).

The quantity of total fat differs to some extent in rye and wheat bran. While rye bran contains approximately 3.9−4.6 % of total fat, the total fat content in wheat bran is about 5.6 %. Since the majority of previous research has focused on the extraction, analysis and purification of alkyresorcinols (**Fig. 1.17**), there are not much data regarding rye bran oil composition. Rye has a different alkyresorcinol homologue distribution and C17:0/C21:0 ratio of ~1, while for spelt wheat the ratio is ~0.1, and ~0.01 for durum wheat (Andersson et al., 2014). Franciso (2005) (Francisco et al., 2005a, 2005b) used supercritical CO_2 to extract alkyresorcinols from rye bran but due to its amphiphilic character, pure CO_2 was not efficient for this process. To increase extraction efficiency, an additional co-solvent (ethanol) was added. Later this process was advanced by supplementing it with the chromatographic purification procedure (Dey & Mikhailopulo, 2009). In another study, SC-CO_2 was compared with ethyl acetate extraction; however, no significant differences were found (Landberg et al., 2007). It was also suggested to use intact kernels instead of milled ones because the former yielded the same amount of alkylresorcinols and was less diluted with other substances. Purified fractions were applied in bioactive emulsions protecting apples against *Penicillium expansum*.(Deya et al., 2013). When used in animal feed, alkylresorcinols can reduce growth and feed consumption in rats and swine (Weiringa et al., 1967).

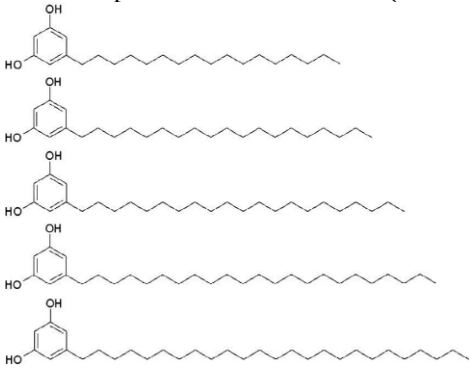


Figure 1.17. 5-n-alkylresorcinols found in rye bran, with odd numbered alkyl chains in range C17:0-C25:0 (from top to bottom) (Landberg et al., 2014)

Rye bran contains phytosterols and phytostanols which are important cholesterol-lowering components, as well as very high levels of ferulic acid and much smaller amounts of *p*-coumaric acid. As in wheat, ferulic acid in rye is mainly attached to arabinoxylan through ester linkages. Rye bran also contains much higher levels of lignans (syringaresinol, medioresinol, pinoresinol, secoisolariciresinol, matairesinol and lariciresinol) compared to wheat bran (Landberg et al., 2014).

1.5.2. Blackcurrant

Blackcurrant (*Ribes nigrum* L.) is a deciduous shrub common in Europe, Asia and North America. It belongs to *Grossulariaceae* family and *Ribes* species (Chrzanowski, 2008). It grows up to 1–2 m tall and produces purple-black, sweet, aromatic, edible seed-containing berries that can grow up to 12 mm in diameter (Gopalan et al., 2012; Nour, Trandafir, & Cosmulescu, 2014). *Ribes nigrum* is a seasonal fruit and must either be consumed rapidly or processed because of its high perishability. About 15−20 % of blackcurrant berries are consumed fresh while the rest are processed commercially into different products, such as juice, alcoholic beverages, jams, jellies, liqueurs, or as a color or flavor ingredient for yogurt and other dairy products.

During blackcurrant juice production, high amounts of valuable residue, or pomace, made of seeds, skins, stalks, stems and leaves of the plant remain. If these by-products are disposed directly into the soil and landfill, they can create serious environmental and ecological problems due to their low pH value. Furthermore, they are prone to microbial spoilage because of their high water activity (Talcott, 2007). Technological changes implemented in the production of fruit juices have made it possible to obtain fruit pomace of considerably dry weight, often above 40 % (Kosmala et al., 2014; Rój & Dobrzyńska-Inger, 2009). Blackcurrant berry pomace is a by-product of great interest to the food industry, since the available carbohydrates, phenolic compounds and pigments can be used either as food ingredients or functional constitutes for enhancing the functionality of food products. Blackcurrants can prevent the adsorption and growth of viruses and can act as disinfectant against some bacteria. Furthermore, their extract can be used either as disinfectant or a component in gargle products, candies or juices as a mild or natural prophylactic against diverse infections (Ikuta et al., 2012).

After pressing the juice, the pomace can be air-dried and selectively sieved out leaving the seedless pomace consisting of skins, stalks and leaves. If this process is omitted, the pomace will contain a high amount of seeds. The seeds are rich in oil which can be retrieved using solvent extraction or employing combinations of solvent extraction with cold pressing. Blackcurrant seed oil contains linoleic acid (44.6 mg/g oil) as the major fatty acid, followed by lower amounts of α- (12.2 mg/g oil) and γ-linolenic acids (12.6 mg/g oil), oleic acid (9.3 mg/g oil) and palmitic acid (5.9 mg/g oil). In comparison to seed extract, the pomace extract, contains tangible amounts of nutritionally important fatty acids, phytosterols and policosanols; however, along with the beneficial constituents, the extraction produces some undesirable components, such as wax esters of policosanols and long-chain fatty acids. As a result, further purification and treatment steps may be required (Dobson et al., 2012). The seed oil extracted from the *Ribes* species is rich in α-linolenic, γ-linolenic and stearidonic acids and they can be metabolized into precursors of anti-inflammatory eicosanoids which produce positive health effects. The composition and gene expression level of the seed oil are highly dependent on the plant species and the stage of development (Vuorinen et al., 2016).

Blackcurrant berries are rich in polyphenolic compounds (500–1342 mg/100 g of fresh fruit) which have antioxidant and cardio-protective properties (Moyer et al.,

2002; Rosenblat et al., 2010). During juice production, the processes of enzymatic depectinisation and pasteurization might reduce the concentration of polyphenolic compounds leaving a large amount of these compounds in the pomace. A positive effect on health can only be seen when sufficient amounts of these polyphenols are consumed, which is difficult to achieve through consuming fresh fruit or common commercial products alone. By utilizing by-products, it is possible to produce food additives with an adequate amount of these valuable compounds (Sojka et al., 2009). Previous experiments with rabbits have showed that polyphenol-rich extract from blackcurrant pomace ingested at relatively high amounts may be a useful therapeutic supplement in the reversal of dysfunctions related to obesity and its complications (Jurgoński et al., 2014).

Blackcurrant fruit are known to have a high content of anthocyanin (160−411 mg/100 g of fresh fruit) and they can act as chemo-preventive agents for human liver cancer (Bishayee et al., 2011; Moyer et al., 2002). Dephinidin-3-glucoside, dephinidin-3-rutinoside, cyaniding-3-glucoside and cyaniding-3-rutinoside are the main anthocyanins which constitute the main phenol class (up to 90%) of blackcurrant pomace and blackcurrant press residue (Kapasakalidis, Rastall, & Gordon, 2006). Besides anthocyanins, *Ribes nigrum* berries contain hydroxycinnamic acids, including derivatives of *p*-coumaric acids, and myricetin, quercetin, kaempferol glycosides as well as a small amount of isorhamnetin (Anttonen & Karjalainen, 2006; Sójka & Król, 2009).

Since blackcurrant pomace contains significant amounts of polysaccharides, it is a great source of dietary fiber. These valuable compounds are usually prepared from dried pomace mostly by physical methods, like sieving and grinding combined with aqueous extraction. A combined extraction of phenols and dietary fiber is very promising in the future of functional food production (Figuerola et al., 2005; Kosmala et al., 2010). The main dietary fiber components (in descending order) determined in previous experiments are lignin, hemicellulose, cellulose and pectin. The amount of lignin and hemicellulose accounted for 84 % of the total dietary fiber of the analyzed pomace (Nawirska & Kwaśniewska, 2005).

2. MATERIALS AND METHODS

2.1. Research objects and pretreatment

Sowing rye (*Secale cereale* L.) and plain wheat (*Triticum aestivum* L.) bran were donated by AB "Kauno Grūdai" (Kaunas Lithuania) and stored in bags at <20 °C in a dry ventilated room. Before the extraction, the bran were ground using an ultra-centrifugal rotor mill Retsch ZM 200 (Retsch GmbH, Haan, Germany) with 2.0 mm size sieve at 8000 rpm and separated with different perforation size sieves into fractions according to particle size: > 1 mm, 1−0.5 mm, 0.5−0.23 mm and < 0.23 mm.

Rye bran for supercritical extraction optimization were ground in Retsch ZM 200 laboratory rotor mill at 8000 rpm using different mesh size sieves for obtaining 3 fractions, <0.2 mm, <0.5 mm and <1.0 mm.

Freshly pressed blackcurrant pomace (*Ribes nigrum*) with seeds was kindly donated by a local company "Obuolių namai" (Kaunas, Lithuania). It was immediately dried (-50 °C and 0.5 mbar) in a Freeze Drying Plant Sublimator 4x5x6 (Zirbus Technology, Bad Grund/Harz, Germany) and ground in Retsch ZM 200 at 8000 rpm using a 0.2 mm sieve. The powdered pomace was stored in hermetically sealed glass jars in a dark and dry room until extraction and fractionation.

2.1.1. Reagents

Analytical grade hexane, acetone and methanol were acquired from Chempur (Piekary Śląskie, Poland) and food grade ethanol was bought from company Strumbras (Kaunas, Lithuania). HPLC grade solvents for chromatographic analyses as well as 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), [2,2-azobis(2-methyl-propionamidine) dihydro-chloride] (AAPH), Folin-Ciocalteu phenol reagent (2M), 3,4,5-trihydroxybenzoic acid (gallic acid), 2,4,6-tripyridyl-*s*-traizine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), tocopherol standards, aluminum trichloride hydrate and ferric chloride ($FeCl_3$) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Carbon dioxide (99.9%) and nitrogen (99.9%) were obtained from Gaschema (Jonava region, Lithuania).) and Fluorescein (FL) sodium salt was bought from Fluka Analytical (Buchs, Switzerland). Randomly methylated β-cyclodextrin (RMCD) (Trappsol, pharmacy grade) was purchased from CTD Holdings, Inc. (High Springs, FL, USA), sodium acetate from Acros Organics (Geel, Belgium), potassium iodide (KI) from Chempur (Piekary Slaskie, Poland) and sodium sulphate (Na_2SO_4) from Eurochemicals (Vilnius, Lithuania). A mixture of fatty acid methyl esters (FAME) was from Supelco Analytical (Bellefonte, PA, USA). Viscozyme® L (cellulotic enzyme mixture; ≥ 100 FBGU/g) was obtained from Sigma Aldrich (Bornem, Belgium); CeluStar XL (xylanase enzyme preparation; 15.000 – 17.500 xylanase units/g) from Dyadic International, Inc. (FL, USA) and diatomaceous earth (DE) dispersant was from Dionex (Sunnyvale, CA, USA).

2.2. The preparation of extracts

The properties of wheat and rye bran (< 0.23 mm particle size) were evaluated by performing Soxhlet extraction in an automated extractor EZ100H (Behr Labor-Technik, Düsseldorf, Germany) as a standard technique AOAC (Chemists, 1995), using hexane and acetone. 20 grams of ground bran were loaded into a cellulose thimble and inserted into the inner tube of the apparatus. The rate of extraction was 1 cycle per 5 min. The Soxhlet extraction residue was then re-extracted with methanol/water ($MeOH/H_2O$; 80:20 v/v) (Anwar et al., 2010) at 25 °C for 90 min in closed vials using the shaker. All extractions were performed sequentially with the same bran sample after the previous solvent was evaporated. These extracts were compared to the results obtained by PLE extraction system. Soxhlet extraction with hexane was compared to the results obtained by supercritical fluid extraction system. The solvent was removed in a rotary vacuum evaporator Büchi Rotavapor R-210 (Haan, Germany) at 40 °C and the residue was weighed on analytical balances. All extractions were replicated three times.

Blackcurrant pomace (< 0.2 mm particle size) was extracted using conventional solvent extraction techniques. 20 g of pomace or its residue were loaded into a cellulose thimble, inserted into the inner tube of the Soxhlet apparatus and then continuously extracted with *n*-hexane for 4 hours. The residue was used for further extraction with acetone at the same conditions. The solvents were evaporated in a rotary vacuum evaporator at 40 °C and the residues were finally dried in an incubator at 50 °C. At the final stage, ethanol/water (EtOH/H_2O; 70:30 v/v) (Abozed et al., 2014) extraction was applied to the residue after acetone extraction. 20 grams of the material was placed in a 500 mL Erlenmeyer flask, 200 mL of solvent was added and the sample was shaken for 2 hours in an orbital shaker. The supernatant was collected using vacuum filtration and the extraction procedure was repeated for the second time combining the supernatants. Ethanol was evaporated using a rotary evaporator at 40 °C, and water was removed by freeze-drying.

2.2.1. Pressurized liquid extraction

PLE was performed in a pressurized liquid extraction apparatus Dionex ASE 350 (Sunnyvale, CA, USA). The sample was placed in a Dionex stainless-steel extraction cell (2.9 mm diameter) which was equipped with a stainless steel frit and a cellulose filter at the ends of the cell to avoid solid particles in the collection vial. During all performed extractions, the cells were preheated for 5−7 min to ensure that the sample reached thermal equilibrium at 10.3 MPa pressure and desired temperature before static extraction in 3 cycles. A flush volume of 100 % of the cell was used; finally, the cell was purged with nitrogen for 60 s to collect the extract in the collection vial. Organic solvents were removed from the extracts in the rotary vacuum evaporator at 40 °C and the residue was finally dried in an incubator at 50 °C. If the solvent contained water, it was removed by freeze drying. The extracts were weighed on analytical balances and stored at -20 °C until antioxidant activity measurements or other analysis. The extractions were replicated at least three times.

Raw wheat and rye bran (> 1 mm, 1−0.5 mm, 0.5−0.23 mm and < 0.23 mm particle size) were extracted consecutively applying different polarity solvents, namely hexane, acetone and a mixture of methanol/water (MeOH/H_2O; 80:20 v/v). The 20 g sample of ground bran was mixed with 2 g of diatomaceous earth and placed in a 66 mL cell. The extraction temperature was kept at constant, 80 °C and each extraction cycle was 5 min (total time – 15 min).

Rye bran material (< 0.2 mm particle size), after SC-CO_2 extraction at optimal conditions, was used to extract with the PLE system. The 12 g sample of ground bran was placed in a 34 mL cell for extractions with acetone at different temperatures (70, 100, 130 °C) and static extraction durations (11, 26, 41 min). Central composite design was applied to achieve the highest yield. After acetone extraction at optimal conditions (130 °C, 41 min), rye bran residue was used to extract with ethanol/water mixture (EtOH/H_2O; 70:30 v/v). An additional sample which was obtained using lower temperature (70 °C) but prolonged extraction time (80 min) was also used for the extraction with the same ethanol mixture. Diatomaceous earth was added to the samples (1:1 ratio) before EtOH/ H_2O

extraction. The extraction conditions for both solvents were kept the same (130 °C, 41 min and 80 °C, 80 min).

After SC-CO_2 extraction at optimal conditions, dried blackcurrant residue (< 0.2 mm particle size) was mixed with diatomaceous earth and extracted consecutively applying pure ethanol and water. The extraction temperature was 70 ºC (EtOH) and 130 ºC (H_2O); the extraction consisted of 3 cycles, 5 min each. These extractions were executed using 34 mL extraction cells.

2.2.2. Supercritical carbon dioxide extraction

SC-CO_2 optimization experiments were performed in a laboratory extraction system Helix (Applied Separations, PA, USA) using 50 mL stainless steel extraction vessel, 14 mm in inner diameter and 320 mm in length. The temperature of the extraction vessel was controlled by a surrounding heating jacket. A schematic diagram of the equipment is presented in **Fig. 2.1**. To prevent the system from clogging, the sample was placed between two layers of cotton wool. The volume of CO_2 was measured by a digital mass flow meter in standard liters per minute (SL/min) at a standard state (P_{CO_2}=100 kPa, T_{CO_2}=20°C, ρ_{CO_2}=0.0018 g/mL) and the CO_2 flow was kept constant for all experiments – 2 SL/min. The process consisted of static (10 min) and dynamic extraction steps. The extracts were collected into glass bottles and kept at -20 °C temperature before analysis.

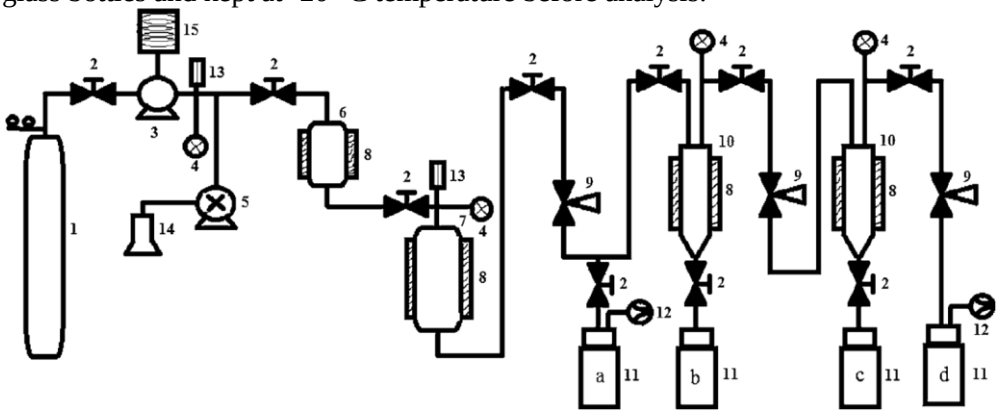


Figure 2.1. The principle schematic diagram of SC-CO_2 system: 1 − CO_2 gas cylinder; 2 – on-off valve; 3 – CO_2 pump; 4 – pressure gauge; 5 – co-solvent pump; 6 – CO_2 vessel; 7 – extraction vessel; 8 – heating jacket; 9 – back pressure regulator; 10 – collection vials for different fractions (a, b, c, d); 12 – gas flow meter; 13 – safety valve; 14 – co-solvent reservoir; 15 – CO_2 pump cooler (Kraujalis & Venskutonis, 2013b)

The following conditions were set to optimize rye bran (< 0.2 mm particle size) extraction: the pressure was between 25 and 55 MPa, the temperature range was 30–70 °C and dynamic extraction time varied from 60 to 120 min. The extraction vessel was filled with 10 g of ground rye bran. SC-CO_2 at optimal conditions was up-scaled in a pilot system (Applied Separations, PA, USA) using 3650 g of bran flour and a 10 L stainless steel extraction vessel. For exhaustive extraction in this system, static and dynamic extraction times were prolonged to 30 min and 210 min, respectively. The ratio of sample mass to extraction solvent in

laboratory-scale apparatus was 0.04, while in the pilot-scale system this ratio was adjusted to 0.05 by varying the extraction duration and carbon dioxide flowrate. The pilot-scale system contains CO_2 regeneration system thus it is possible to reduce the CO_2 gas consumption.

The conditions for blackcurrant pomace (< 0.2 mm particle size) extraction optimization were set as follows: the pressure ranged from 30 to 55 MPa, temperature was between 30 and 60 °C and dynamic extraction time was from 60 to 150 min. A 50 mL extraction vessel was filled with 12 g of ground blackcurrant pomace. The optimal conditions (45 MPa, 60 °C, 120 min) were tested and the kinetic curve was obtained in the Helix extraction system, using a bigger extraction cell (500 mL) and 100 g of ground material. For exhaustive extraction in this system, the static extraction time was prolonged to 30 min and the dynamic time was extended to 240 min.

2.2.3. Enzyme-assisted extraction

Optimal EAE parameters for obtaining the highest yield of soluble substances were set as follows: E/S ratio 6 % v/w (72 Fungal β-Glucanase Units (FBGU)/10 g pomace), temperature 40 °C, pH 3.5 and extraction time of 7 hours. Blackcurrant pomace residue after PLE with ethanol was additionally treated with another xylanolytic enzyme mixture, CeluStar XL, under the same conditions in order to compare its efficiency to Viscozyme® L. The EAE was carried out by a modified procedure used by Kapsaklidis (Kapasakalidis et al., 2006): 10 g of pomace were weighted in a 250 mL polyethylene flat-bottom centrifugation bottle and suspended in 100 mL (50 nM) sodium acetate buffer; then, the enzyme was added at a required E/S ratio. Control samples Blank A (pomace with buffer), Blank B (enzyme with buffer) and Blank C (buffer) were prepared simultaneously. The prepared mixtures were incubated in thermostatically controlled shaker VWR (Radnor, PA, USA) at 800 rpm. The enzyme extraction was terminated by immersing the sample bottle in a boiling water bath for 10 min, followed by a rapid cooling and centrifugation (9000 rpm, 10 min) with the Velocity18r centrifuge (Dynamica Scientific Ltd., United Kingdom). The resulting supernatants (water-soluble fraction) and solid residue (water-non-soluble fraction) were collected, freeze-dried and kept in a freezer (-20 °C) prior the analysis. The amount of water-soluble fraction was determined gravimetrically.

2.3. The evaluation of antioxidant activity

2.3.1. Oxygen radical absorbance capacity assay

The ORAC was performed following Prior's method (Prior et al., 2003). It uses fluorescein as a probe and reactive oxygen species (ROS) generated by the decomposition of AAPH which can quench the fluorescent signal of fluorescein. The reaction was carried out in 75 mM phosphate buffer (pH 7.4); a stock solution of fluorescein was ready and the samples were prepared by dissolving plant extracts in methanol. Extracts obtained in SC-CO_2 extraction system were analyzed using L-ORAC assay for lipophilic fraction (Huang et al., 2002). 10 mg of oil were dissolved in 1 ml of 7% RMCD solution (acetone:water; 50/50 v/v) to solubilize the

antioxidants and diluted to the required concentration. The RMCD solution (7 %) was used as a blank. The prepared samples, the blank, or trolox solutions (25 μL) and fluorescein (150 μL; 14 μM/L) solutions were placed in the 96-well clear flat-bottom black opaque microplates. The microplates were sealed and incubated for 15 min at 37 °C. After incubation, AAPH solution as a peroxyl radical generator (25 μL; 240 mM) was added manually with a multichannel pipette.

The QUENCHER procedure was applied to measure antioxidant activity of solid samples directly, without any extraction by mixing them with free radicals (Gökmen et al., 2009). For the procedure, 10 mg of sample or cellulose (blank) were transferred to a centrifugation tube, mixed with 150 μL of PBS solution and 900 μL of fluorescein solution, vortexed for 15 s, shaken at 250 rpm for 30 min in the dark and centrifuged (1960×g for 5 min). The optically clear supernatant (175 μL) was transferred to the 96-well black opaque microplates, incubated for 15 min at 37 °C, followed by a rapid addition of 25 μL of AAPH solution.

After adding the AAPH solution, the microplate was immediately placed in the FLUOstar Omega (BMG Labtech GmbH, Offenburg, Germany) fluorescent reader. The plate with the samples was shaken prior to each reading and the fluorescence measurements (excitation wavelength 485 nm; emission wavelength 510 nm) were taken every 66 s, in a total of 120 cycles. At least three independent measurements were performed for each sample. Raw data were analyzed using the Mars (BMG Labtech GmbH, Offenburg, Germany) software. Fluorescein and AAPH solutions were prepared fresh daily. The antioxidant curves (fluorescence over time) were normalized and the area under the fluorescence decay curve (AUC) was calculated with:

$$AUC = 1 + \sum_{i=1}^{i=120} \frac{f_i}{f_0}; \quad (6)$$

where f_0 is the initial fluorescence at 0 min and f_i is the fluorescence at time i.

The final ORAC values were calculated using a regression equation between the trolox concentration and the AUC. A series of trolox solutions were used as a reference antioxidant in a wide concentration range according to the sample activity. The antioxidant activity was expressed in trolox equivalent (TE) antioxidant capacity per gram extract or per gram of dry weight (DW).

2.3.2. Total phenolic content by Folin-Ciocalteu's assay

The TPC was determined using the Singleton and Rossi's method (Singleton et al., 1965) with slight modifications. 10 μL of appropriate dilutions of the extracts or gallic acid solutions were oxidized with 190 μL of Folin-Ciocalteau's reagent (2 M) solution in distilled water (1:13). The reagents were mixed in 96-well microplate, allowed to sit for 3 min and then neutralized with 100 μL of 7 % Na_2CO_3. The mixture was vortexed for 90 min and the absorbance was measured at 765 nm in the FLUOstar Omega reader (BMG Labtech, Offenburg, Germany).

For the QUENCHER procedure, 10 mg of sample or cellulose (blank) were transferred to centrifugation tube, mixed with 150 μL of distilled water, 750 μL of Folin-Ciocalteu's reagent previously diluted with distilled water (1:9, v/v). After 3 min of reaction, 600 μL of Na_2CO_3 solution were added. The prepared mixtures

were vortexed for 15 s, shaken at 250 rpm for 2 h in the dark and centrifuged (1960×g for 5 min); the absorbance of optically clear supernatant was measured.

The TPC was calculated using gallic acid calibration curve and expressed in milligram gallic acid equivalents per gram of extract or per gram of DW.

2.3.3. $ABTS^{\bullet+}$ decolourization assay

Trolox equivalent antioxidant capacity (TEAC) assay was used to determine the radical scavenging capacity (RSC) of extracts as described by Re et al. (1999) but with slight modifications. The pH of phosphate buffered saline (PBS) solution (8.18 g NaCl, 0.27 g KH_2PO_4, 1.78 g Na_2HPO_4 x 2 H_2O and 0.15 g KCl in 1 L of distilled water) was adjusted to 7.4 with NaOH, while the working solution of $ABTS^{\bullet+}$ was produced by reacting 50 mL $ABTS^{\bullet+}$ (2 mM) stock solution with 200 µL potassium persulphate ($K_2S_2O_8$ (70mM)); the mixture was kept in the dark for 14−16 h before measurements. The working solution was further diluted with PBS to obtain the absorbance of 0.800±0.030 at 734 nm. Extracts, blank solutions, or trolox solutions (3 µL) were reacted with 300 µL of $ABTS^{\bullet+}$ solution in a 96-well microplate. After 30 min, the absorbance was analyzed at 734 nm in a FLUOstar Omega reader. Trolox solutions of various concentrations were used for the calibration curve.

For the QUENCHER procedure, 10 mg of sample or cellulose (blank) were transferred to a centrifugation tube, mixed with 25 µL of MeOH and 1500 µL of working $ABTS^{\bullet+}$ solution. The prepared mixtures were vortexed for 15 s, shaken at 250 rpm for 2 h in the dark and centrifuged (1960×g for 5min); the absorbance of optically clear supernatant was measured at 734 nm.

Antioxidant activity of ABTS•+ was calculated with the formula:

$$I = [\frac{(A_{blank} - A_{sample})}{A_{blank}}] \times 100; \quad (7)$$

where: I – $ABTS^{\bullet+}$ inhibition, %; A_{blank} – absorption of blank sample (t = 0 min); A_{sample} – absorption of tested extract solution (t = 30 min).

The TEAC values were calculated from the calibration curve and the RSC values were expressed in TE per gram of extract or per gram of DW.

2.3.4. $DPPH^{\bullet}$ scavenging assay

$DPPH^{\bullet}$ assay is based on radical scavenging by the antioxidant which results in a decrease in absorbance at 515 nm (Brand-Williams et al., 1995). The stable $DPPH^{\bullet}$ radical was dissolved in methanol to prepare the working solution and to obtain the absorbance of 0.700±0.030 AU at 515 nm. Briefly, 7.5 µL of extracts, blank or trolox solutions were mixed in the 96-well microplate with 300 µL of $DPPH^{\bullet}$ solution. The measurements were taken after 45 min at 515 nm using a FLUOstar Omega reader.

For the QUENCHER procedure, 10 mg of sample or cellulose (blank) were transferred to a centrifugation tube, mixed with 500 µL of MeOH and 1000 µL of a working $DPPH^{\bullet}$ methanol solution. The prepared mixtures were vortexed for 15 s, shaken at 250 rpm for 2 h in the dark, centrifuged (1960×g for 5min) and the absorbance of optically clear supernatant was measured.

The results were calculated as in the $ABTS^{\bullet+}$ assay. A series of trolox solutions of various concentrations were used for calibration and the final results were expressed in TE per gram of extract (μM TE/g) or per gram of DW.

2.3.5. Ferric reducing antioxidant power assay

The FRAP assay was performed using the Benzie and Strain's method (Benzie & Strain, 1996). The FRAP solution was prepared in a proportion of 10:1:1 of acetate buffer (300 mM) (pH 3.6), 2,4,6-tripyridyl-*s*-triazine (TPTZ) (10 mM) in HCl (40 mM) and $FeCl_3$ (20 mM), respectively. The FRAP solution was incubated at 37 °C for 30 min before mixing it with the appropriate dilution of the sample. 50 μL of extract, 150 μL of distilled water, standard, or blank (methanol) and 1500 μL FRAP reagent were mixed and left in a dark room for two hours. The absorbance was measured at 593 nm using Spectronic Genesis spectrophotometer.

For the QUENCHER procedure, 10 mg of sample or cellulose (blank) were transferred to a centrifugation tube, mixed with 50 μL of MeOH, 150 μL of distilled water and 1500 μL of freshly prepared FRAP reagent. The prepared mixtures were vortexed for 15 s, shaken at 250 rpm for 2 h at 37 °C, centrifuged (1960×g for 5 min) and the absorbance of the optically clear supernatants was measured at 593 nm. The results were expressed in TE per gram of extract or per gram of DW.

2.3.5. The evaluation of oil stability

Blackcurrant oil obtained at optimal conditions was used to test its stability by accelerated oil stability tests in Oxipres (ML, Oxipres, Mikrolab, Denmark) and Metrohm 873 Biodiesel Rancimat apparatus (Metrohm Ltd., Switzerland) at 110 °C using 5 g of oil (Baranauskiene et al., 2014). Using the Oxipres method, the system was filled with oxygen at 0.5 MPa, while using the Rancimat stability testing, air was purged through the samples at the rate 20 L/h. Several commercial oils were used for comparison.

2.4. Chromatographic analysis

2.4.1 The determination of fatty acid profile by gas chromatography

Fatty acid composition of SC-CO_2 extract was analyzed by gas chromatography (GC) after converting fatty acids into their methyl esters (FAME) using BF_3 catalyst following the official AOAC method (Chemists, 1995). FAMEs were analyzed on the HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) equipped with a flame ionization detector and 100 m long 0.25 mm inside diameter, 0.20 μm film thickness fused silica capillary column SPTM−2560 (Supelco, Bellafonte, PA, USA). The parameters for the analysis were set as follows: injection temperature was 220 °C; the temperature of the detector was 240 °C; split ratio 100:1; the oven temperature was programed in three ramps, from 80 °C to 135 °C at 4 °C/min, from 135 °C to 185 °C at 4 °C/min and from 185 °C to 240 °C at 4 °C/min, and held for 5 min. Helium was used as carrier gas at a flow rate of 20 cm^3/s. The compounds were identified by comparing their retention times with those of a commercial FAME mixture. Triplicate GC runs were performed and the results are presented as a mean (RSD value did not exceed 10 %).

2.4.2 High performance liquid chromatographic determination of tocopherols

Perkin Elmer Series 200 HPLC system equipped with a C30 reverse-phase column and a thermostat at 30 °C (particle size 5 μm, 250×4.6 mm) was used for tocopherol analysis applying isocratic elution with acetonitrile:methanol:dichlormethane (72/22/6, v/v/v) (Gruszka & Kruk, 2007) with slight modifications. The injection volume was 20 μL and the flow rate was 1 mL/min. Tocopherols were detected using a fluorescence detector at 290 nm excitation and 330 nm emission. The analytes eluted in 20 min: α-tocopherol at 12.2 min, β-tocopherol at 10.6 min, γ-tocopherol at 10.2 min and δ-tocopherol at 8.9 min. Tocopherols were identified by comparing the retention time of peaks to those of standard solutions which were prepared at different concentrations using a mobile phase. Extracts were prepared dissolving in the mobile phase at final concentration of 1 mg/mL. The calibration curves (peak area versus injected amount) were used to determine the concentration of tocopherols in the samples. The analyses were performed three times (RSD value did not exceed 10 %).

2.5. The experimental design

Response surface methodology (RSM) using central composite design (CCD) (Box & Wilson, 1951) was used to carry out the extract yield optimization experiments. Our goal was to find the settings for the design variables that maximize the yield of the extract. In order to maximize the yield, the extract has to be sufficiently soluble in supercritical solvent. The solubility strongly depends on the pressure and temperature (Sovová, 2012). Therefore both of these parameters were included in SC-CO_2 extraction optimization experiments. Dynamic extraction time was chosen as the third important process variable. In the PLE system the pressure is kept constant, thus temperature and extraction time were chosen as optimization variables.

The complete design for SC-CO_2 extraction consisted of 20 experimental runs with 8 factorial points, 6 axial points and 6 center points. The multiple regression equation was used to fit the second-order polynomial equation (3) based on the experimental data. The complete design of PLE with acetone consisted of 15 experimental runs with 4 factorial points, 4 axial points and 5 center points. Furthermore, according to experimental data, the multiple regression equation was used to fit the second-order polynomial equation. All experimental variables and their variation levels are presented in **Table 2.1.**

Table 2.1. Independent variables and their variation levels for the central composite design

Optimization of SC-CO_2 extraction of rye bran			
Variation levels	Variables		
	Extraction pressure, MPa	Extraction temperature, °C	Dynamic extraction time, min
Low level (-1)	25	30	60
Max level (+1)	55	70	120
Medium level (0)	40	50	90

Star point (-α)	14.77	16.36	39.55
Star point (+α)	65.23	83.64	140.45

Optimization of PLE with acetone of rye bran after SC-CO_2 extraction at optimal conditions

Variation levels	Variables	
	Extraction temperature, °C	Extraction time, min
Low level (-1)	70	11
Max level (+1)	130	41
Medium level (0)	100	26

Optimization of SC-CO_2 extraction of blackcurrant pomace

Variation levels	Variables		
	Extraction pressure, MPa	Extraction temperature, °C	Dynamic extraction time, min
Low level (-1)	30	30	60
Max level (+1)	55	60	150
Medium level (0)	42.5	45	105
Star point (-α)	21.48	19.77	29.32
Star point (+α)	63.52	70.23	180.68

For SC-CO_2 extraction of rye bran, three independent variables and their variation levels were chosen based on previously reported data: pressure (25–55 MPa), temperature (30–70 °C) and dynamic extraction time (60–120 min). It should be noted that in our study the selected maximum level of pressure was considerably higher than the highest level (35 MPa) used in previously performed studies with rye bran (Dey & Mikhailopulo, 2009; Francisco et al., 2005a, Francisco et al., 2005b; Landberg et al., 2007). Francisco et al. (2005a) have concluded that extract yield was proportional to pressure when it is higher than 30 MPa.

Rye bran residue obtained at optimal SC-CO_2 extraction conditions was used for the extraction optimization in PLE with acetone as a solvent. Two independent variables at three variation levels were chosen: temperature (70–130 °C) and extraction time (11–41 min). This experiment was carried out to test how temperature and extraction time affects the extraction yield when acetone is used. Since axial (or star) points are not located outside the original factor range, this plan is face centered.

Exactly the same variables were chosen for optimizing the blackcurrant pomace SC-CO_2 extraction. The conditions for extraction were set as follows: extraction time was between 60 and 150 min, pressure was from 30 to 55 MPa and the extraction temperature ranged between 30 and 60 °C. To the best of our knowledge, there are only a few works on blackcurrant extraction with SC-CO_2 and it has mainly been applied for the seed fraction (Bártlová et al., 2006; Rój & Dobrzyńska-Inger, 2009; Soares et al., 2007; Sovová et al, 2001); the highest applied pressure was around 30 MPa. SC-CO_2 extraction optimization was carried out mainly on apple peel (J. Li, Guo, & Li, 2015), raspberry (Kryzeviciute, Kraujalis, & Venskutonis, 2016), olive (Akay et al., 2015), and tomato (Zuknik et al., 2012) pomaces.

All experiments were performed at least in triplicate and in random order. Proper randomization of the experiment allows minimizing the effects of possible

extraneous factors. Thus, any potential bias that can result from the drift in the applied measuring instrument is minimized (Burdick, Borror, & Montgomery, 2005).

All the results were analyzed using StatEase DesingExpert Trial computer program (Stat-Ease Inc., Minneapolis, MN, USA). Statistical significance of the model and variables were determined at 5 % probability level ($p<0.05$). The adequacy of our estimated model has been determined by evaluating the 'lack of fit' coefficient and the Fisher test value (F-value) obtained from analysis of variance (ANOVA).

2.6. Statistical data evaluation

All analyses were carried out at least in triplicate and the results were expressed as a mean ± standard deviation (SD). Differences among means were determined by one-way ANOVA using the Statgraphics Plus 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA) statistical package. Fisher's least significant difference (LSD) was used to determine significant difference among the samples at 5 % probability level ($p<0.05$). Correlation coefficients were calculated between each of the variables using MS Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

3. RESULTS AND DISCUSSION

3.1. The extraction yield and antioxidant properties of wheat and rye bran extracts obtained with pressurized liquid extraction

The amount of information available with regards to wheat and rye bran antioxidant properties is small. To the best of our knowledge, PLE technique has not been applied until now for the preparation of extracts and their antioxidant capacity assessment. Rye bran extraction studies are remarkably scarcer, since rye grain is commonly used only in the Nordic European countries. Wheat and rye brans were divided into several fractions by their particle size and extracted with different polarity solvents. The efficiency of the extraction processes was evaluated applying a systematic evaluation of antioxidant potential by determining extract properties via several antioxidant activity assays.

3.1.1. The extraction yield of wheat and rye bran

Rye and wheat bran were extracted by consecutively applying solvents of different polarity, namely hexane, acetone and methanol with water. The results of these extractions are presented in **Table 3.1**. A higher extract yield was obtained from rye bran (9.83–10.56 %) than from wheat bran (5.61–7.11 %), especially using methanol. Rye bran has a higher content of polar substances. The particle size has a significant effect on the extract yield ($p>0.05$); the yield of methanol extracts of wheat bran increases significantly by decreasing the particle size. The same tendency can be observed for rye bran extracts obtained with hexane. It should be noted that the PLE solvent was unable to penetrate fully through the sample matrix.

In order to obtain the highest yield, the contact surface with the solvent has to be increased by decreasing the particle size.

Table 3.1. The extraction yield of wheat and rye bran obtained by PLE

Rye bran	Solvent	Yield, %	Wheat bran	Solvent	Yield, %
> 1 mm	HE	2.25±0.02[a]	> 1 mm	HE	2.51±0.05[c]
	AC	0.93±0.05[b]		AC	1.19±0.02[c]
	MeOH/H_2O	9.89±0.01[c]		MeOH/H_2O	5.77±0.02[b]
0.5–1 mm	HE	2.44±0.07[b]	0.5–1 mm	HE	2.65±0.07[e]
	AC	0.92±0.01[b]		AC	1.24±0.01[d]
	MeOH/H_2O	9.83±0.04[b]		MeOH/H_2O	5.61±0.07[a]
0.23–0.5 mm	HE	2.61±0.04[c]	0.23–0.5 mm	HE	2.08±0.03[a]
	AC	0.86±0.01[a]		AC	0.94±0.01[b]
	MeOH/H_2O	9.96±0.04[d]		MeOH/H_2O	6.15±0.09[d]
< 0.23 mm	HE	2.91±0.02[d]	< 0.23 mm	HE	2.29±0.03[b]
	AC	0.87±0.01[a]		AC	0.96±0.02[b]
	MeOH/H_2O	10.56±0.06[e]		MeOH/H_2O	7.11±0.07[e]
CE (< 0.23 mm)	HE	2.90±0.08[d]	CE (<0.23 mm)	HE	2.61±0.05[d]
	AC	0.88±0.03[a]		AC	0.88±0.03[a]
	MeOH/H_2O	9.53±0.61[a]		MeOH/H_2O	5.93±0.10[c]

The values are presented as means ± standard deviations (n=3); the mean values followed by different letters are significantly different (p<0.05) between one type of solvent and different particle sizes; CE – conventional extraction; HE – hexane; AC – acetone; MeOH/H_2O – methanol/water (80:20 %).

It should be noted that methanol which was used in this study for laboratory scale experiments, although being a highly toxic solvent, is allowed for all uses by the Directive 2009/32/EC (2009); however, its maximum residue limits in the extracted foodstuff or food ingredient should not exceed 10 mg/kg. From this perspective, ethanol might be recommended as a safer solvent, since it possesses physical and dissolving properties similar to those of methanol.

3.1.2. The effect of solvent on antioxidant activity indicators of rye and wheat bran extracts

There are many assays for the assessment of antioxidant properties of plant extracts. The majority of them are based on hydrogen atom or single electron transfer reactions. It has been concluded that ORAC, TPC and one of the hydrogen/electron transfer assays should be used for the representative evaluation of antioxidant properties of food and food ingredients (Huang et al., 2005). Following this recommendation, all of these methods were applied for assessing antioxidant potential of bran extracts in this study. Antioxidant potential indicators were expressed in grams of extracts and recalculated for 1 g of bran dry weight. Both values are useful as they show the activity of extracts obtained by different solvents and reveal the potential of the whole bran material, respectively. For instance, the extracts may be strong antioxidants, while their yields may be very low; as a contrast, extracts which are less active antioxidatively but high in yield would

isolate a larger part of antioxidants from the whole bran matrix. The results of rye and wheat bran antioxidant activity after PLE are shown in **Table 3.2**.

The TPC values are in a wide range for both rye (12.5±0.2–53.0±0.1 mg GAE/g) and wheat (13.2±0.1–40.2±0.1 mg GAE/g) bran; the acetone extracts of rye and wheat bran contain the highest TPC, (up to 53.1±0.1 mg GAE/g in rye and up to 40.2±0.1 mg GAE/g in wheat) followed by hexane and methanol extracts; however, due to notably higher yields isolated with methanol, the latter solvent seems to be the most effective in extracting phenolic compounds from the sample matrix. Thus, the amounts of TPC isolated with methanol (0.83−1.42 mg GAE/g DW) are about 2 times higher comparing with hexane (0.31−0.68 GAE/g DW) and 3 times higher comparing with acetone (0.33−0.48 mg GAE/g DW). Methanol was previously reported as the most effective solvent in extracting phenolic compounds from wheat bran using conventional (0.24 mg/g) and microwave-assisted solvent extraction (0.47–0.49 mg/g) (Oufnac et al., 2007); however, the results were expressed in catechin equivalents. In another study (Wang, Sun, Cao, Tian, & Li, 2008), ultrasound-assisted extraction was optimized and the extracts isolated with 64 % ethanol contained the highest content of TPC (3.12 mg GAE/g wheat bran), followed by 70 % methanol (~2.1 mg GAE/g wheat bran) and 70 % acetone (~1.9 mg GAE/g wheat bran); however, in this case the bran material was not fractionated with consecutive extractions.

$DPPH^{\bullet}$ scavenging values vary from 16.77±0.19 to 85.50±0.21 μM TE/g for rye bran extracts and from 4.4±0.1 to 71.2±0.2 μM TE/g for wheat bran extracts. The values expressed in μM TE/g DW are 0.50–2.09 and 0.10–1.99, respectively. Again, methanol has extracted the highest amount of $DPPH^{\bullet}$ scavengers both from rye (1.69–2.09 μM TE/g DW) and wheat (1.44–1.99 μM TE/g DW) bran. In general, wheat bran extracts possess lower RSC in $DPPH^{\bullet}$ assay comparing to rye bran extracts. The values of RSC in $ABTS^{\bullet+}$ assay of bran extracts are in an even broader range, from 29.6±0.1 to 218.4±0.2 for rye, and from 22.5±0.1 to 212.3±0.2 μM TE/g for wheat. In this assay, particularly high amounts of radical scavengers have been isolated with methanol, up to 19.62 and 15.09 μM TE/g DW for rye and wheat, respectively. Methanol wheat bran extracts were previously reported as possessing the highest antioxidant capacity, 0.042 μM TE/g for conventional and 0.064–0.072 μM TE/g for microwave-assisted extraction (Oufnac et al., 2007); however, the values obtained in this study are substantially lower comparing to our results.

The variations in ORAC values measured for the extracts isolated with different solvents are not so extreme comparing with $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays. ORAC values for rye and wheat bran extracts are in the ranges of 2131.4±0.3–2955.7±0.3 and 2049±0.36–2787±0.31 μM TE/g, respectively. However, due to differences in extract yields, the ORAC values expressed in μM TE/g DW are in a wider range, 25.71–233.38 and 23.0–160.63 for rye and wheat respectively. Due to higher extract yields, methanol has isolated a higher content of constituents demonstrating ORAC, up to 233.38 and 160.63 μM TE/g DW from rye and wheat bran, respectively.

Table 3.2. The results of rye and wheat bran antioxidant activity: $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC are expressed as μM TE/g extracts and μM TE/g DW plant material; TPC is expressed in mg GAE/g extract and mg GAE/g DW plant material

Sample	Solvent	TPC		$ABTS^{\bullet+}$		$DPPH^{\bullet}$		ORAC	
		Extract	DW	Extract	DW	Extract	DW	Extract	DW
Rye bran (> 1 mm)	HE	22.8±0.05^{a}	0.51^{a}	34.0±0.03^{a}	0.77^{a}	27.0±0.13^{d}	0.61^{c}	2166±0.21^{a}	48.74^{a}
	AC	46.8±0.16^{a}	0.43^{a}	113.7±0.12^{a}	1.06^{a}	54.5±0.16^{a}	0.51^{a}	2826±0.23^{a}	26.28^{c}
	MeOH/H_2O	14.4±0.11^{b}	1.42^{c}	198.4±0.13^{d}	19.62^{d}	21.2±0.18^{b}	2.09^{d}	2131±0.31^{a}	210.76^{a}
Rye bran (0.5-1 mm)	HE	23.8±0.15^{c}	0.58^{b}	32.2±0.16^{a}	0.79^{b}	20.7±0.18^{c}	0.50^{a}	2188±0.33^{a}	53.38^{b}
	AC	51.7±0.15^{b}	0.48^{c}	130.0±0.23^{a}	1.20^{c}	66.0±0.19^{b}	0.61^{b}	2861±0.31^{ab}	26.32^{c}
	MeOH/H_2O	14.4±0.18^{b}	1.41^{c}	162.3±0.19^{c}	15.95^{c}	18.7±0.11^{a}	1.83^{c}	2146±0.37^{a}	210.93^{a}
Rye bran (0.23-0.5 mm)	HE	23.2±0.09^{ab}	0.60^{c}	32.2±0.07^{a}	0.84^{c}	19.3±0.12^{b}	0.50^{a}	2524±0.43^{b}	65.89^{c}
	AC	51.7±0.17^{b}	0.44^{a}	136.4±0.15^{a}	1.17^{b}	73.4±0.21^{c}	0.63^{b}	2881±0.33^{ab}	24.78^{a}
	MeOH/H_2O	12.8±0.15^{a}	1.27^{a}	140.7±0.23^{b}	14.01^{b}	17.0±0.19^{a}	1.69^{a}	2155±0.40^{a}	214.62^{a}
Rye bran (< 0.23 mm)	HE	23.5±0.16^{bc}	0.68^{d}	29.6±0.09^{a}	0.86^{d}	17.9±0.17^{a}	0.52^{b}	2647±0.43^{c}	77.02^{d}
	AC	53.0±0.10^{c}	0.46^{b}	218.4±0.19^{b}	1.90^{d}	85.5±0.21^{d}	0.74^{c}	2955±0.26^{b}	25.71^{b}
	MeOH/H_2O	12.5±0.19^{a}	1.32^{b}	89.9±0.23^{a}	9.49^{a}	16.7±0.19^{a}	1.77^{b}	2210±0.33^{b}	233.38^{b}
Wheat bran (> 1 mm)	HE	18.7±0.10^{d}	0.47^{c}	36.8±0.05^{ab}	1.28^{c}	27.0±0.11^{d}	0.51^{d}	2564±0.26^{a}	64.35^{c}
	AC	34.0±0.16^{a}	0.40^{c}	81.3±0.11^{a}	0.97^{a}	36.7±0.17^{a}	0.44^{a}	2173±0.28^{a}	25.86^{b}
	MeOH/H_2O	14.4±0.09^{b}	0.83^{a}	89.9±0.13^{a}	7.47^{a}	24.9±0.10^{a}	1.44^{a}	2049±0.36^{a}	118.22^{a}
Wheat bran (0.5-1 mm)	HE	16.5±0.07^{c}	0.44^{b}	48.6±0.07^{b}	1.29^{c}	12.5±0.10^{c}	0.33^{c}	2635±0.32^{b}	69.83^{d}
	AC	37.1±0.17^{b}	0.46^{d}	127.5±0.17^{b}	1.58^{c}	51.9±0.22^{b}	0.64^{c}	2325±0.21^{b}	28.83^{d}
	MeOH/H_2O	15.7±0.11^{c}	0.88^{b}	157.2±0.19^{b}	8.82^{b}	26.6±0.18^{b}	1.49^{a}	2074±0.38^{a}	116.34^{a}
Wheat bran (0.23-0.5 mm)	HE	14.8±0.13^{b}	0.31^{a}	36.5±0.09^{ab}	0.76^{b}	8.9±0.03^{b}	0.18^{b}	2673±0.31^{c}	55.59^{a}
	AC	40.2±0.06^{c}	0.38^{b}	160.7±0.19^{c}	1.51^{b}	61.7±0.24^{c}	0.58^{b}	2448±0.29^{c}	23.01^{a}
	MeOH/H_2O	16.5±0.16^{d}	1.01^{d}	209.9±0.21^{c}	12.91^{c}	27.3±0.11^{c}	1.68^{b}	2128±0.29^{b}	130.88^{b}
Wheat bran (< 0.23 mm)	HE	13.4±0.13^{a}	0.31^{a}	22.5±0.09^{a}	0.52^{a}	4.4±0.01^{a}	0.10^{a}	2673±0.40^{c}	61.22^{b}
	AC	34.2±0.06^{a}	0.33^{a}	190.8±0.19^{d}	1.83^{d}	71.2±0.22^{d}	0.68^{d}	2787±0.31^{d}	26.75^{c}
	MeOH/H_2O	13.2±0.14^{a}	0.94^{c}	212.3±0.22^{d}	15.09^{d}	28.0±0.13^{d}	1.99^{c}	2259±0.38^{c}	160.63^{c}

The values are presented as means ± standard deviations (n=3); the mean values followed by different letters are significantly different (p<0.05) using one type of solvent to analyze different particle sizes. DW – dry weight; HE – hexane; AC – acetone; MeOH/H_2O – methanol/water (80:20 %).

3.1.3. The effect of particle size on antioxidant activity indicators of rye and wheat bran extracts

It is well known that particle size has some influence on the extraction process. The same quantity of smaller particle size material has a larger total particle surface for diffusion of soluble analytes into the solvent. However, the extraction depends on both the properties of solid material and solvent, therefore the particle size should be individually selected for different plant materials. In this study, 4 different size fractions were used, >1, 0.5−1.0, 0.23−0.5 and <0.23 mm; some interesting tendencies have been observed regarding the effects of particle size on the antioxidant activity of extracts.

The effect of particle size on TPC is not substantial for all extracts, although significant differences have been calculated for many values listed in **Table 3.2**. Nevertheless, some interesting tendencies have been observed. For instance, in the rye hexane extract, the TPC values have been noticed to be statistically different but the analysis has not shown any dependence on the particle size. In wheat hexane extracts, the TPC seems to be inversely proportional to the particle size. The compositional peculiarities of rye and wheat bran (different proportions of tocopherol, phenolic acid and alkyresorcinols) may be the reason for these differences.

The effects of particle size on $ABTS^{\bullet+}$ scavenging also reveal some interesting tendencies. For instance, the RSC of $ABTS^{\bullet+}$ of rye acetone extracts has gradually increased from 113.7±0.12 to 218.4±0.19 μM TE/g when the particle size decreased from >1 to <0.23 mm; however, the RSC of $ABTS^{\bullet+}$ of rye methanol extracts at the same conditions decreased from 198.4±0.13 to 89.9±0.23 μM TE/g. As long as the extraction is performed sequentially and methanol is applied after hexane and acetone, these findings may be explained by considering that in case of smaller particles acetone extracts more $ABTS^{\bullet+}$ scavengers; consequently, the residue contains a lower amount of antioxidants available for extraction with methanol.

A similar tendency may be observed for rye extracts in $DPPH^{\bullet}$ assay, although the differences are less notable. The results obtained with different size wheat bran fractions for acetone extracts are generally in agreement with the results obtained for rye bran: the smaller the particle size is, the stronger $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging. Contrary to rye bran, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging capacities of methanol extracts have also increased by reducing the particle size. This may depend both on the differences in rye and wheat bran matrices and the composition of soluble antioxidants. A fairly lower RSC of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ of hexane extracts isolated from the smallest particle size wheat fraction may be regarded as another characteristic peculiarity of wheat bran; the recorded differences between rye bran hexane extracts isolated from different particle size fractions are less pronounced, although the tendencies are similar to wheat bran.

The variations in ORAC values both between the solvents and between the fractions are less substantial; however, it has been observed that ORAC values are higher for the extracts isolated from smaller particles which is in agreement with previously reported results showing that increasing the ratio of surface area to mass

of the material under extraction may result in higher ORAC values by 80 % (Brewer et al., 2014). Other authors have indicated a positive effect of grinding on the antioxidant capacity of wheat bran fractions and it has linearly correlated with the specific surface of the comminuted material (Rosa et al., 2013).

3.1.4. The overall antioxidant activity and correlation between different determination methods

The overall antioxidant activity potentials of rye and wheat bran expressed in a sum of values of all extracts obtained with different solvents are presented in **Figure 3.1**. It is obvious that rye bran possesses a higher TPC (2.31–2.47 mg GAE/g DW) than wheat bran (1.58–1.78 mg GAE/g DW); however, the variations in TPC between different particle size fractions for the same type of bran have not been considerable (**Fig. 3.1, *a***). Rather unexpected results have been obtained from the overall RSC of $ABTS^{\bullet+}$ for rye bran (**Fig. 3.1, *b***): a decreasing particle size has resulted in a decrease in the sum of $ABTS^{\bullet+}$ scavenging capacities from 21.45 to 12.25 μM TE/g DW. With regards to wheat bran, on the other hand, the sum of RSC of $ABTS^{\bullet+}$ has increased from 9.72 to 17.44 μM TE/g DW. The differences most likely depend on the compositional peculiarities of rye bran which greatly differ from wheat bran (Apprich et al., 2014; Nordlund et al., 2013). These findings clearly indicate that particle size for plant material under extraction should be carefully selected in every case individually. The differences in the sums of RSC values in $DPPH^{\bullet}$ assay are not so prominent for all bran samples: for rye and wheat bran they are in the ranges of 2.82–3.21 and 2.39–2.77 μM TE/g DW, respectively.

It is known that the $ABTS^{\bullet+}$ assay is more suitable both for lipophilic and hydrophilic extracts, while $DPPH^{\bullet}$ assay is more applicable for hydrophilic extracts. This study uses different polarity solvents, including nonpolar hexane dissolving lipophilic compounds and high polarity methanol/water which is better for extracting hydrophilic antioxidants. This may explain the differences for the overall antioxidant potential in the used assays of RSC. Rye bran possesses a higher antioxidant potential in ORAC assay than wheat bran; moreover, the smallest particle size fractions produce higher total ORAC values both for rye and wheat (**Fig. 3.1, *c***). It has already been mentioned that some previous studies have also determined an increase in ORAC values of wheat bran by decreasing the particles used for the extraction (Brewer et al., 2014; Rosa et al., 2013), while such studies with rye bran have not been performed until now.

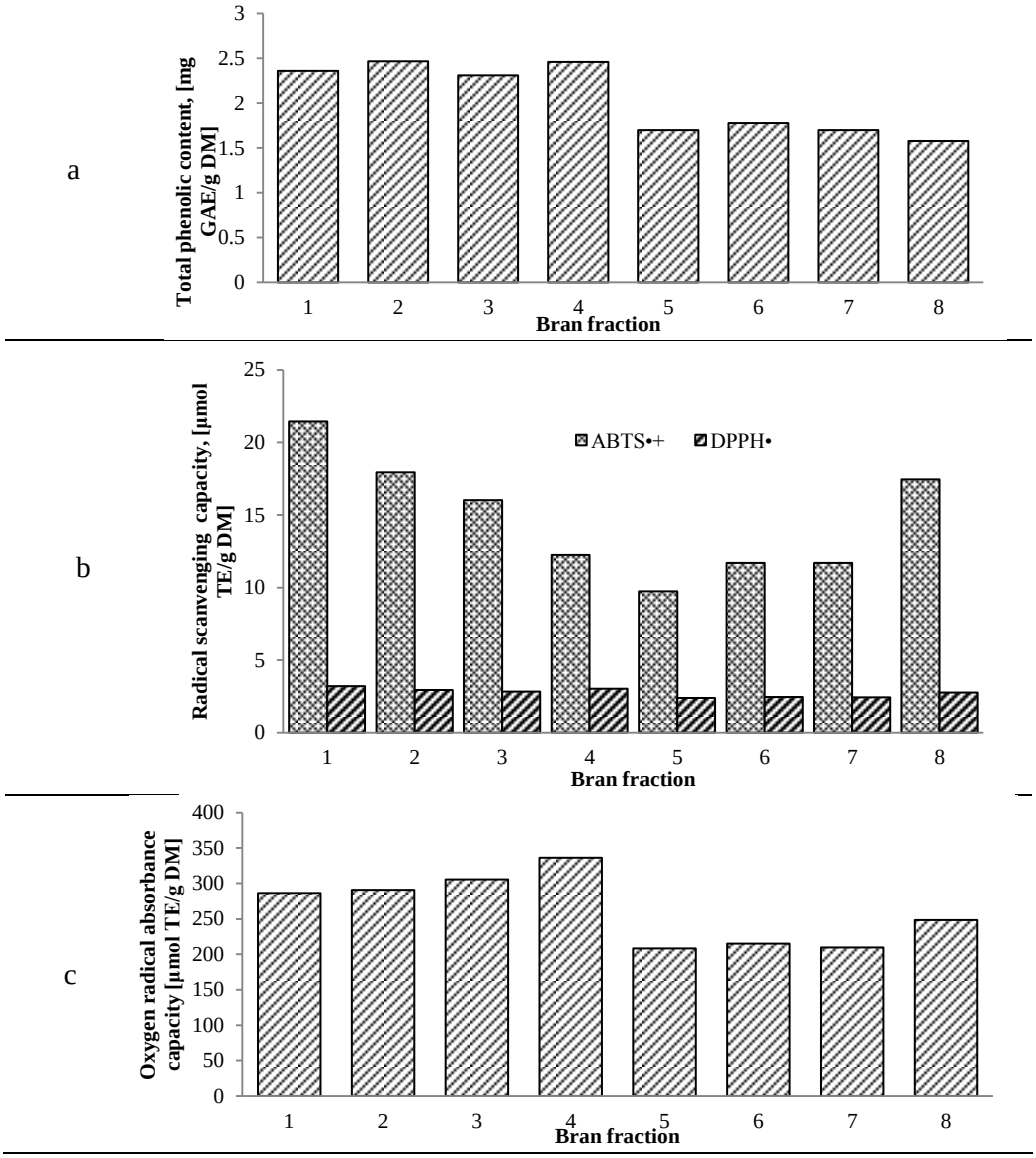


Figure 3.1. The accumulative antioxidant activity values of rye (RB) and wheat (WB) bran expressed as sums of total phenolic content (a); $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging capacity (b); oxygen radical absorbance capacity (c); 1 – RB > 1 mm; 2 – RB 0.5-1 mm; 3 – RB 0.23-0.5 mm; 4 – RB < 0.23 mm; 5 – WB > 1 mm; 6 – WB 0.5-1 mm; 7 – WB 0.23-0.5 mm; 8 – WB < 0.23 mm

Systematic studies of antioxidant activity indicators using 4 assays applied to 4 different size fractions and using 3 different polarity solvents has resulted in numerous values. Therefore, it is interesting to assess if there are correlations between different assay methods. For instance, numerous studies have demonstrated strong positive correlations between TPC values and RSC of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. The correlation coefficients for different antioxidant assays are summarized in

Figure 3.2. It is observed that there are strong correlations between different antioxidant activity assays when the values are expressed for plant material DW (0.91−0.99 for rye and 0.84−0.97 for wheat bran). However, when antioxidant activity values are expressed per gram of extract, the correlations are more complicated. For instance, correlation coefficients for TPC–ABTS$^{\bullet+}$, TPC–DPPH$^{\bullet}$ and ABTS$^{\bullet+}$–DPPH$^{\bullet}$ methods are strongly positive and somewhat similar to wheat and rye bran samples. However, the correlations with ORAC values are not so consistent. For instance, TPC–ORAC (0.924) and DPPH$^{\bullet}$–ORAC (0.85) values are highly correlating for rye bran, while no correlation has been noticed for wheat bran between these assays; the coefficients are 0.1 and 0.02, respectively. The correlations between ABTS$^{\bullet+}$–ORAC values are 0.19 for rye and -0.40 for wheat bran. These findings reveal the complexity of the composition of antioxidatively active constituents in rye and wheat bran. In comparison to many green botanicals, bran contains more lipophilic and amphiphilic antioxidants and therefore their distribution in the extracts isolated by different polarity solvents may be of truly unique character. Comprehensive studies of individual constituents as well as their antioxidative properties are needed for obtaining more exhaustive information on the properties of rye and wheat bran extracts.

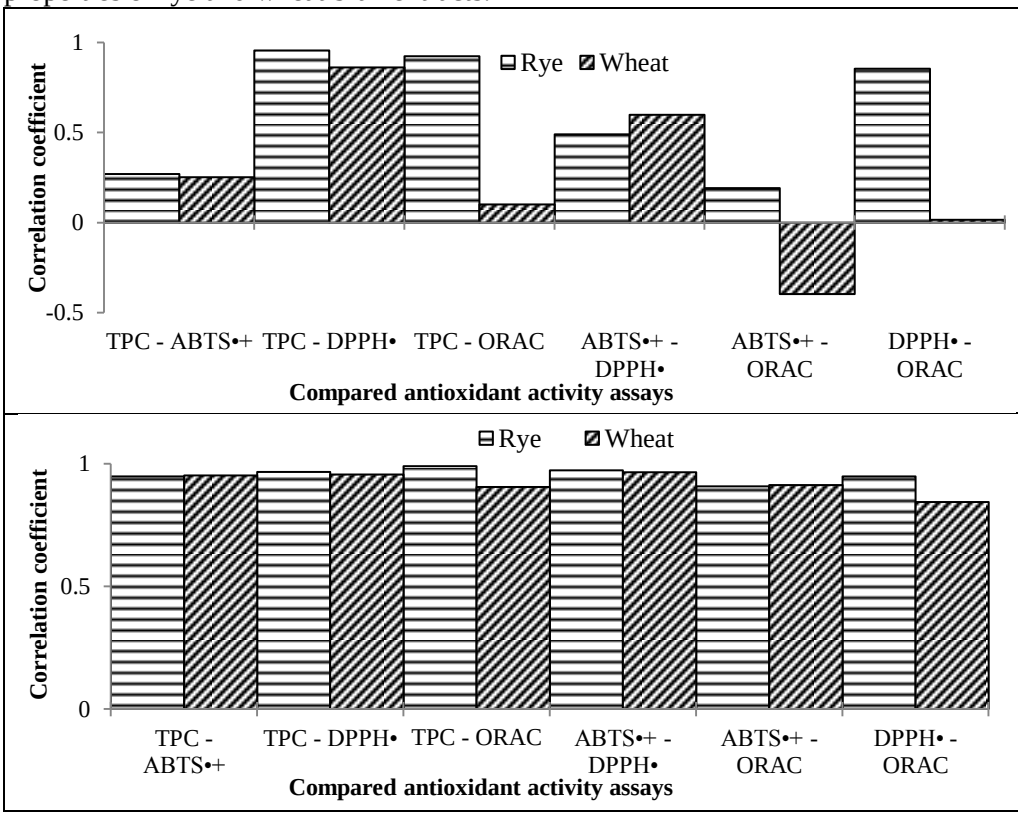


Figure 3.2. The correlation coefficients between different antioxidant measurement assays of different rye and wheat bran milling fractions; top – in g of extract; bottom – in g dry plant material

3.2. The optimization of high pressure extraction of rye bran using mathematic modelling tools

Valuable compounds from cereal bran have been extracted using organic solvents and PLE system (**Fig. 3.3)**. This extraction procedure is more efficient as compared to various conventional extraction procedures, which are usually time consuming and require large volumes of solvents which are difficult to remove with extract purification (Agil et al., 2012; Mattila, Pihlava, & Hellström, 2005; Zarnowski & Suzuki, 2004; Zhou & Yu, 2004). In order to obtain cleaner extracts, supercritical CO_2 extraction can be used.

The majority of studies have been focused on the isolation of alkylresorcinol-rich fraction, while the optimization of SC-CO_2 parameters for the isolation of lipophilic fraction from rye bran has not been performed previously. Many reports have demonstrated that the application of RSM-CCD for the optimization of extraction parameters may significantly improve process efficiency in terms of product yield (Kemzuraite et al., 2014; Kraujalis & Venskutonis, 2013a). Properly selected parameters may give substantially higher extract yields from rye bran.

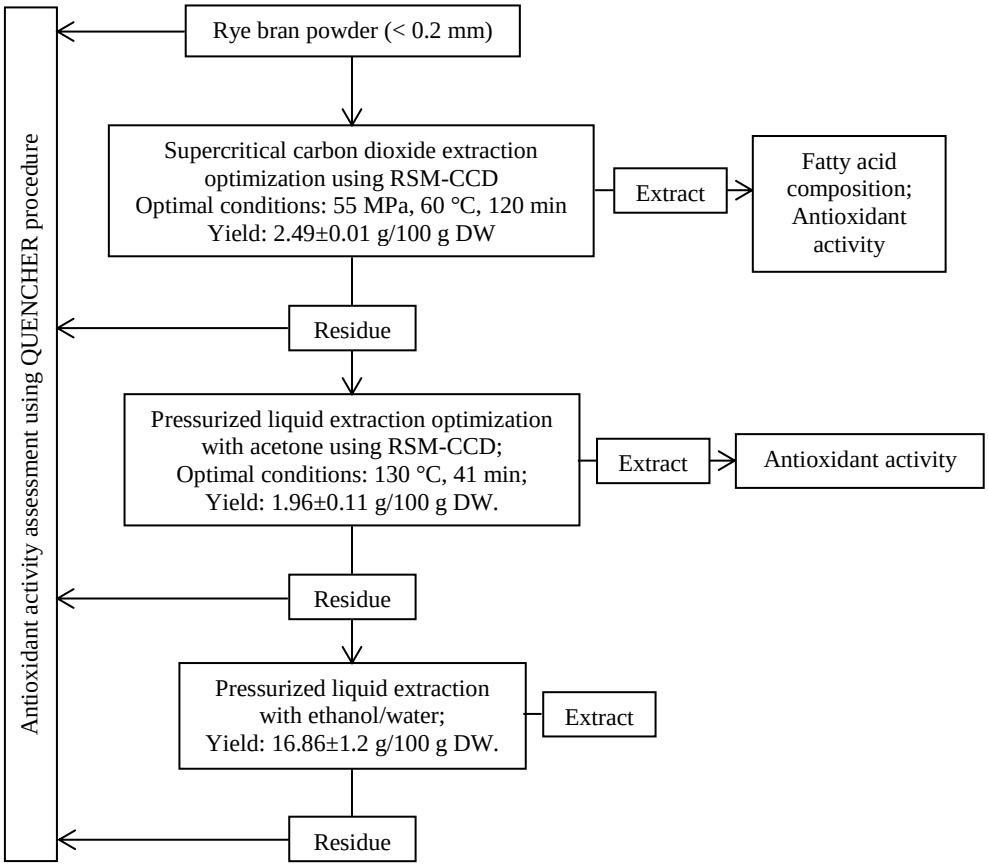


Figure 3.3. The biorefining scheme of rye bran by SC-CO_2 and PLE

3.2.1 The optimization of the supercritical fluid extraction of rye bran

The first part of the experiment evaluates the SC-CO_2 kinetics using different pressures (15, 35, 55 MPa) at a constant 70 °C temperature. The obtained results are presented in **Figure 3.4**. It is obvious that using a lower pressure (15 and 35 MPa), the major part of the extract is isolated during the first 30 min of dynamic extraction. Nevertheless, at the highest pressure (55 MPa) the yield of extracts continuously increases up to 90–120 min of process time thus the extraction optimization has been performed in a range from 60 to 120 min of dynamic extraction.

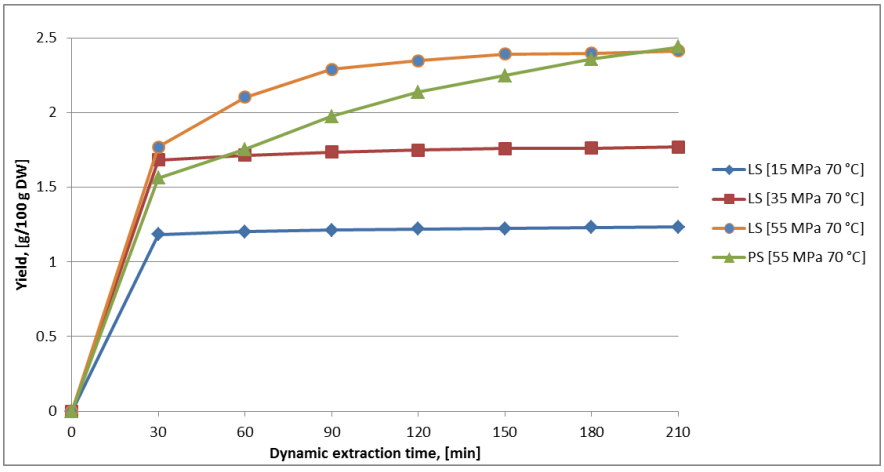


Figure 3.4. The influence of dynamic extraction time on the rye bran extract yield (LS – laboratory scale extractor; PS – pilot scale extractor; RSD values did not exceed 10 %)

Previous experiments with PLE have revealed that rye and wheat bran particle size has significant influence on the extraction yield. Subsequently, the effect of rye bran particle size has also been evaluated prior to optimization by applying the previously reported SC-CO_2 conditions (35 MPa, 70°C) which give the highest yield of 32.6 mg/g DW (with ethanol as entrainer) (Francisco et al., 2005a, Francisco et al., 2005b). The highest yield without entrainer is achieved (24.4±0.1 mg/g DW) by using the most finely ground fraction (<0.2 mm fraction). The yields from other fractions are: 20.3±0.1 mg/g DW for <0.5 mm fraction and 19.5±0.1 mg/g DW for <1 mm fraction. Consequently, the finest fraction has been selected for optimization experiments.

Table 3.3. CCD parameter values and the results of rye bran extract optimization; 1–8 are experimental runs, 9–14 are axial point runs and 14–20 are central point runs

	Coded factor			Uncoded factor			
Run	Extraction pressure	Extraction temperature	Dynamic extraction time	Extraction pressure, MPa	Extraction temperature, °C	Dynamic extraction time, min	Yield, g/100 g DW
1	-1	-1	-1	25	30	60	1.43±0.05
2	1	-1	-1	55	30	60	1.67±0.09
3	-1	1	-1	25	70	60	1.85±0.04
4	1	1	-1	55	70	60	2.16±0.05
5	-1	-1	1	25	30	120	1.68±0.04
6	1	-1	1	55	30	120	1.92±0.09
7	-1	1	1	25	70	120	2.03±0.02
8	1	1	1	55	70	120	2.49±0.01
9	-1.682	0	0	14.77	50	90	1.49±0.09
10	1.682	0	0	65.23	50	90	2.16±0.06
11	0	-1.682	0	40	16.36	90	1.53±0.07
12	0	1.682	0	40	83.64	90	2.27±0.09
13	0	0	-1.682	40	50	39.55	1.70±0.07
14	0	0	1.682	40	50	140.45	2.23±0.05
15	0	0	0	40	50	90	2.06±0.05
16	0	0	0	40	50	90	2.13±0.04
17	0	0	0	40	50	90	2.18±0.02
18	0	0	0	40	50	90	2.08±0.03
19	0	0	0	40	50	90	2.20±0.08
20	0	0	0	40	50	90	2.08±0.08

Results are expressed as mean ± standard deviation of triplicate determinations.

The results of SC-CO_2 extraction optimization are presented in **Table 3.3**. The application of CCD to optimize three independent variables (pressure, temperature and dynamic extraction time) at three different levels allowed obtaining response surface plots (**Fig. 3.5**) which show the effect of these variables on rye bran extract yield. The graphs were obtained by keeping one of the variables constant (coded as zero level) while varying the other two variables and predicting the response. Three-dimensional (3D) graphs reveal the interactions between different parameters and evaluate their importance on the extract yield.

At a constant 40 MPa pressure (**Fig. 3.5, *A***), the extract yield increases if the process temperature and the duration of extraction is increased, although the effect of temperature is more important than the effect of extraction time. Extending the extraction time from 60 to 120 min increases the yield by 10 %, while increasing the temperature from 30 to 70 °C increases the yield by 30 %. Analogous dependencies can be noticed at constant 50 °C temperature (**Fig. 3.5, *B***): increasing the extraction time from 60 to 120 min results in additional 0.3 % of extract while increasing the

pressure from 25 to 45–55 MPa results in 0.4 % increment of extract. It is also observed that the effect of pressure in the range of 45–55 MPa is negligible. Consequently, the extraction pressure and temperature have been found to be the most important parameters affecting the extract yield; the effects of interactions between these parameters are shown in **Fig. 3.5, *C***.

It is obvious that the increase in both parameter values results in higher extract yields. For instance, at the highest applied temperature (70 °C), an increase of pressure from 25 to 55 MPa results in 0.5 % extract gain. Similar effects on the yield have been observed by increasing the temperature at high pressure levels. These findings may be explained by the enhanced diffusion of CO_2 into the sample matrix and the increased vapor pressure of bran extract oil at higher temperature; whereas when the temperature is raised at lower pressure levels, the CO_2 density decreases resulting in weaker CO_2 diffusion into the oil (Tomita et al., 2014). The yield of rye bran extract varies from 1.43 to 2.49 g/100 g DW (14.32–24.89 mg/g bran DW) using different extraction conditions; other authors have reported considerably smaller yields obtained with pure SC-CO_2, e.g. only ~4 mg/g DW (Francisco et al., 2005a). Adding a co-solvent, ethanol, increases the yields up to ~8–20 mg/g DW (Francisco et al., 2005a), 19 mg/g DW (Dey & Mikhailopulo, 2009) or 30 mg/g DW (Landberg et al., 2007), which is rather similar to the yields obtained in our study using pure CO_2. Conventional solvent extraction with hexane yields 27.89±0.63 mg/g DW, which is only slightly higher than the yield obtained with SC-CO_2.

Considering that the involvement of organic co-solvent makes the process more complex and requires additional solvent removal from the extracts, the optimization of SC-CO_2 in our study has demonstrated the possibilities of increasing extraction efficiency of rye bran by using pure CO_2. The optimal conditions for the highest SC-CO_2 extract yield are 55 MPa, 70 °C and 120 min. The extraction process at these conditions has been upscaled using a pilot extractor with a 10 L vessel. The data shows (**Fig. 3.4**) that the kinetics of the extraction process at optimal parameters in the pilot extractor is slightly different from the kinetics in the laboratory scale extractor; for instance, the extraction yield in the pilot extractor after 120 min of extraction is lower by 10 %; however, at the end of the process (180−210 min), the yields are equal for both extractors.

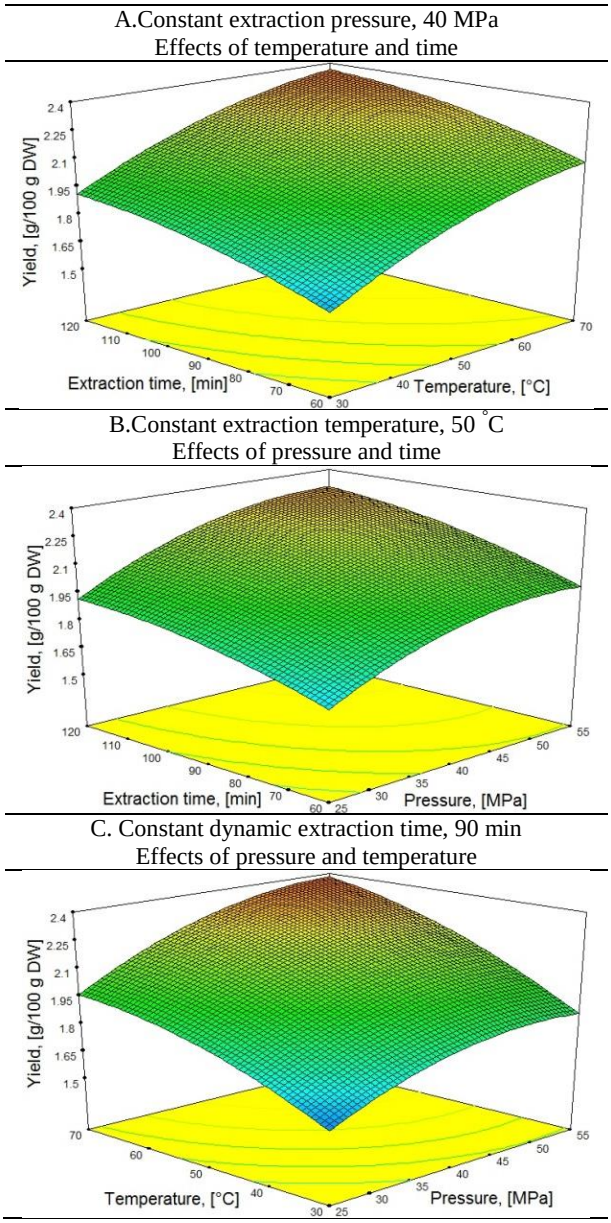


Figure 3.5. Response surface plots for the effects of SC-CO_2 for the yield of rye bran oil

3.2.2 A statistical analysis of rye bran extraction optimization model

This experiment applies RSM with CCD to test three different independent variables: pressure, temperature and dynamic extraction time (P, T and t, respectively). The analysis of variance (ANOVA) of this model is presented in **Table 3.4**. The calculated F-value (59.7) shows that the model is significant according to the Student test (p<0.05). Furthermore, the lack of fit is not significant relative to pure error (p=0.5843). The determination coefficient is R^2=0.9817

(adjusted R^2=0.9653) which is also in reasonable agreement with the predicted R^2=0.9203.

Table 3.4. ANOVA for evaluated response surface quadratic model for rye bran

Source	Sum of squares	df	Mean squares	*F*-ratio	*p*-value
Model	1.6	9	0.18	59.70	<0.0001*
P: Pressure	0.41	1	0.41	137.69	<0.0001*
T: Temprature	0.69	1	0.69	234.54	<0.0001*
t: Time	0.26	1	0.26	89.15	<0.0001*
PT	0.011	1	0.011	3.61	0.0866**
Pt	2.701×10^{-3}	1	2.701×10^{-3}	0.91	0.3628**
Tt	1.125×10^{-6}	1	1.125×10^{-6}	3.786×10^{-4}	0.9849**
P^2	0.15	1	0.15	49.41	<0.0001*
T^2	0.076	1	0.076	25.72	0.0005*
t^2	0.035	1	0.035	11.88	0.0063*
Residual	0.030	10	2.971×10^{-3}		
Lack of fit	0.013	5	2.675×10^{-3}	0.82	0.5843**
Pure error	0.016	5	3.268×10^{-3}		
Cor Total	1.63	19			

* − significant values; ** − non-significant values

The actual and predicted values of the total yield are presented in **Figure 3.6**. The adequate precision (Press) is equal to 27.854 and the ratio greater than 4 is desirable; this indicates an adequate signal and therefore the model can be used to navigate the design space. The adequate precision measure shows the signal to noise ratio which compares the range of the predicted values at the design points to the average prediction error.

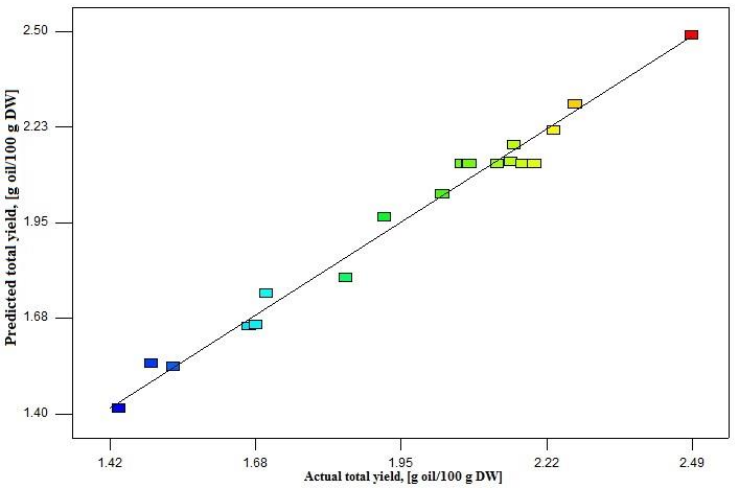


Figure 3.6. A comparison of predicted values and actual values for the extraction yield of rye bran (RSD values did not exceed 10 %)

The results reveal which parameters are significant and the most important in the model. There are six significant model factors – P, T, t, P^2, T^2 and t^2, which can be arranged in the order of importance as follows: temperature > pressure >

extraction time. The results from CCD can be expressed with the following second order polynomial model equation (7):

$$Yield, \% = 2.12 + 0.17 \cdot \mathrm{P} + 0.22 \cdot \mathrm{T} + 0.14 \cdot \mathrm{t} + 0.037 \cdot \mathrm{P} \cdot \mathrm{T} + 0.018 \cdot \mathrm{P} \cdot \mathrm{t} + 3.75 \cdot 10^{-4} \cdot \mathrm{T} \cdot \mathrm{t} - 0.1 \cdot \mathrm{P}^2 - 0.073 \cdot \mathrm{T}^2 - 0.049 \cdot \mathrm{t}^2 \quad (7)$$

3.2.3. The fatty acid composition and antioxidant capacity of rye bran oil

The fatty acid composition is an important characteristic of oil nutritive value; therefore the FA profile of rye bran oil present in SC-CO_2 extract has been analyzed by GC-FID (**Table 3.5**). The dominant FA in rye bran oil is linoleic acid (61.09 %), followed by palmitic (13.74 %), oleic (13.65 %) and linolenic (6.37%) acids. Thus, the total content of unsaturated FA is up to 82 %, while the remaining FAs have been found in considerably lower amounts, 0.07−1 %. To the best of our knowledge, the FA composition of SC-CO_2 extracts of rye bran has not been reported previously, while the FA profile of wheat bran oil isolated by SC-CO_2 is somewhat similar to the FA profile of rye bran investigated in our study; the major FAs of wheat bran oil are linoleic (56 %), oleic (17.4 %) and palmitic (16.9 g/100 g) (Rebolleda et al., 2014). Other studies on SC-CO_2 extracts of wheat bran have also reported the linoleic acid as the major FA followed by the oleic acid (Athukorala, Hosseinian, & Mazza, 2010; Durante et al., 2012; Kwon et al., 2010). The ratio of saturated/unsaturated fatty acids (S/U) is an important factor in evaluating the nutritional value of oil. In the oil obtained at optimal conditions, the S/U ratio is 0.18 which implies that the oil is rich enough in unsaturated fatty acids to be successfully used for dietary enrichment. Rye bran oil has already been applied as a substitute for fat in the production of meatballs (Yilmaz, 2004).

Table 3.5. The fatty acid composition of rye bran oil extracted by SC-CO_2

Fatty acids	SC-CO_2 rye bran oil content, %	Wheat bran oil content, % (Kumar & Krishna, 2013)	Different brand rice bran oil content, % (Krishna, Hemakumar, & Khatoon, 2006)
Pentadecanoic (C15:0)	0.09±0.01	-	-
Palmitic (C16:0)	13.74±0.06	18.9	12.8-21.6
Palmitoleic (C16:1)	0.17±0.01	-	0.0-0.3
Heptadecanoic (C17:0)	0.07±0.01	-	-
Stearic (C18:0)	0.71±0.00	0.4	0.7-4.7
Oleic (C18:1)	13.65±0.08	16.4	32.4-43.4
Linoleic (C18:2)	61.09±0.30	58.6	28.0-53.4
Arachidic (C20:0)	0.14±0.00	-	0.5-1.4
cis-11-Eicosenoic (C20:1)	1.00±0.02	-	-
Linolenic (C18:3)	6.37±0.00	5.5	0.2-1.6
cis-11,14-Eicosadienoic (C20:2)	0.13±0.02	-	-
Behenic (C22:0)	0.09±0.01	-	-
cis-8,11,14-Eicosatrienoic (C20:3)	0.14±0.01	-	-
Erucic (C22:1)	0.23±0.04	-	-
Lignoceric (C24:0)	0.11±0.01	-	-
Nervonic (C24:1)	0.15±0.00	-	-
Saturated	14.95		
Monounsaturated	15.20		
Polyunsaturated	67.61		
Saturated/Unsaturated	0.18		

The results shown are mean values from duplicate analyses and are given in % ±standard deviation (SD).

Cereal bran is known to contain various bioactive molecules, including antioxidants. In addition, the lipid fraction of bran consists mainly of oxidation susceptible polyunsaturated FA. Therefore, it is important to assess the antioxidant properties of rye bran SC-CO_2 extract. The L-ORAC assay has been selected for this purpose, since it is more related to the processes taking place in biological systems than other in vitro assays. The measured ORAC value of rye bran extract obtained at optimal conditions in a pilot scale apparatus is 683.8±45 μM TE/g of extract. In comparison, ORAC values of wheat bran extracted with hexane:dichloromethane (1:1) from the coarse, medium and fine milling fractions are 304.5, 1824.7 and 1787.8 μM TE/g of bran, respectively (Brewer et al., 2014). $DPPH^{\bullet}$ and TPC values of rye bran extract have also been measured and they are 62.28±1.2 μM TE/g of extract and 14.62±0.61 mg GAE/g of extract, respectively. Another study (Andreasen et al., 2001) has concluded that rye bran is a good source of phenolic antioxidants that can have positive health effects.

3.2.4. The optimization of rye bran residue extraction using pressurized liquid extraction

Rye bran residue which was obtained from the pilot scale SC-CO_2 extractor using optimal extraction conditions was used in further biorefining procedures. After removing all lipophilic constituents, more polar solvents, namely acetone and ethanol/water mixture were applied to remove more polar constituents. Since PLE is mainly affected by the temperature and extraction duration, these two independent variables have been optimized using RSM with CCD (**Table 3.6**). The total phenolic content was also measured to test how the extraction conditions affect the antioxidant capacity.

Table 3.6. Parameter values and results of rye bran PLE with acetone optimization; 1–4 are experimental runs, 5–8 axial point runs and 9–13 are central point runs

	Coded factor		**Uncoded factor**		
Run	Extraction temperature	Extraction time	Extraction temperature, °C	Extraction time, min	Yield, g/100 g DW
1	-1	-1	70	11	0.74±0.02
2	1	-1	130	11	1.60±0.02
3	-1	1	70	41	1.25±0.09
4	1	1	130	41	1.96±0.11
5	-1	0	70	26	1.08±0.03
6	1	0	130	26	1.65±0.11
7	0	-1	100	11	1.07±0.08
8	0	1	100	41	1.64±0.03
9	0	0	100	26	1.43±0.09
10	0	0	100	26	1.37±0.12
11	0	0	100	26	1.32±0.12
12	0	0	100	26	1.22±0.09
13	0	0	100	26	1.43±0.08

Results are expressed as mean ± standard deviation of triplicate determinations.

Using PLE system with acetone, the extract yield varies from 0.74±0.02 to 1.96±0.11 g/100 g DW. The highest yield with acetone (1.96±0.11 g/100 g DW) is obtained using 130 °C temperature and extracting for 41 min. The application of CCD for the experiment produces the response surface design which is presented in **Figure 3.7**. These plots show how the two parameters influence rye bran extraction. A larger yield of the extract is produced by increasing the extraction temperature and extraction time. When the temperature is kept at 70 °C, increasing the extraction time from 11 min to 41 min generates almost twice as large a yield of extract (from 0.7 to 1.35 g/100 g DW). A slightly lower but still significant growth can be achieved at high temperatures (above 100 °C); when the extraction is prolonged (41 min), raising the temperature from 70 to 130 °C increases the extract yield from 1.35 to 1.9 g/100 g DW. Similar results can be replicated with lower yield and shorter extraction time. These response surface plots are of great benefit in making the

extraction more efficient; it can be done by increasing the extraction time at lower temperatures or applying high temperature for a short extraction time. For example, 1.35 g/100 g DW yield is obtained just after 41 min at 70 °C. If the extraction temperature is increased to 110 °C, the same yield can be obtained after 11 min.

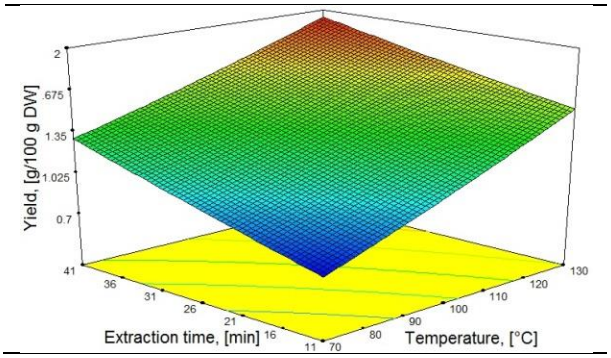


Figure 3.7. The response surface plots for the effects of PLE with acetone on the yield of rye bran extract from residue after SC-CO_2

Both independent variables (T and t) are significant in the evaluated central composite design (**Table 3.7**). They can be arranged by their importance for the extract yield in the following order: extraction temperature > extraction time. ANOVA of the model shows that the model is significant according to the Student test (p<0.05); the lack of fit, however, is not significant. The determination coefficient is R^2=0.9553. An adequate precision of 20.33 indicates an adequate signal of the model, thus it can be used to navigate through the design space. The results from CCD can be used to compose the second-order polynomial model equation (8).

$$Yield, \% = 1.35 + 0.36 \cdot \mathrm{T} + 0.24 \cdot \mathrm{t} - 0.038 \cdot \mathrm{T} \cdot \mathrm{t} + 0.021 \cdot \mathrm{T}^2 + 8.655 \times 10^{-3} \cdot \mathrm{t}^2 \quad (8)$$

Table 3.7. Analysis of variance for the response surface quadratic model

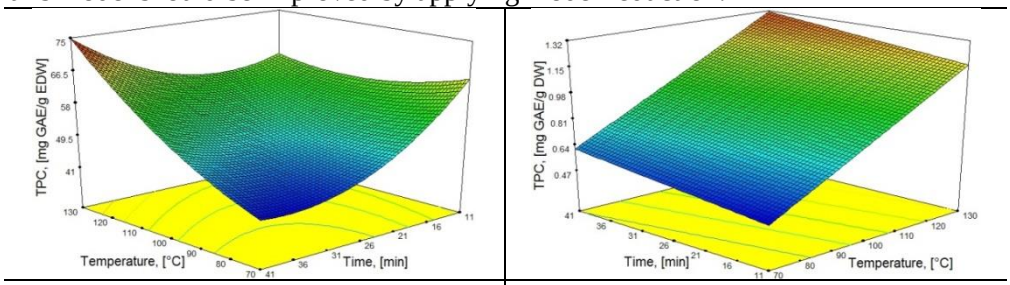
Source	Sum of squares	df	Mean squares	*F*-ratio	*p*-value
Model	1.11	5	0.22	29.91	0.0001*
T: Temprature	0.76	1	0.76	102.35	<0.0001*
t: Time	0.34	1	0.34	46.17	0.0003*
Tt	5.776×10^{-3}	1	5.776×10^{-3}	0.78	0.4076**
T^2	1.178×10^{-3}	1	1.178×10^{-3}	0.16	0.7026**
t^2	2.069×10^{-4}	1	2.069×10^{-4}	0.028	0.8723**
Residual	0.052	7	7.444×10^{-3}		
Lack of fit	0.022	3	7.176×10^{-3}	0.94	0.5007**
Pure error	0.031	4	7.644×10^{-3}		
Cor Total	1.17	12			

* − significant values; ** − non-significant values

Another trial has been applied to test if it is possible to obtain a similar yield (yield at optimal conditions − 1.96±0.11 g/100g DW) by applying a lower temperature (70 °C) but prolonging the extraction time to 40, 60, 80 and 100 min. The experiment has shown that the yield increases until 80 min of extraction, when it reaches a plateau (2.03±0.13 g/100 g DW). These conditions (70 °C, 80 min) have been used for the further analysis with ethanol mixture. The extract yield using the PLE system with ethanol/water mixture is 16.93±0.48 g/100 g DW (at 70°C and 81 min) and 16.86±1.2 g/100 g DW (at 130 °C and 41 min).

3.2.5. The antioxidant activity of rye bran fractions obtained by PLE

In the PLE optimization experiment the total phenolic content has also been measured to assess the extract antioxidant activity and its changes as depending on different extraction conditions (**Fig. 3.8**). The results are presented as response surface plots and expressed for acetone extract dry weight and rye bran dry weight. According to ANOVA, RSM is significant, while the lack of fit is not significant; both of the optimization parameters (temperature and extraction time) have a significant influence on the TPC values. An adequate precision, which is over 4, indicates an adequate signal and thus the model can be used to navigate the design space. The temperature is the most important variable, followed by the extraction time. The model determination coefficients are R^2=0.85 for EDW and R^2=0.95 for DW. This model was used only for screening studies; for a more detailed analysis, this model should be improved by applying model reduction.



TPC expressed for acetone extract dry weight | TPC expressed for rye bran dry weight

Figure 3.8. Response surface plots for total phenolic content in acetone extracts

The figure above presents the changes in the antioxidant capacity through different PLE conditions. When the TPC is expressed for EDW and the extraction temperature is kept at 70 °C, an increase in the extraction time decreases the antioxidant activity. This effect can be caused by extract dilution with less active substances. The opposite result is obtained using a high temperature – 130 °C, where the antioxidant capacity increases while the extraction time is prolonged. A high temperature and 41 min of extraction time produces an extract with the highest total phenol content. Similar results have been obtained in another study, where the highest antioxidant capacity has been obtained using a high temperature (129 °C) (Hossain et al., 2011). The elevated temperature and pressure allows the antioxidant compounds to become more soluble and to achieve a higher diffusion rate therefore the solvent can penetrate the sample matrix more efficiently. When the TPC is

expressed for rye bran dry weight, it is obvious that both variables increase the extraction efficiency. While the extraction time has little effect on the TPC, even a slight increase in the extraction temperature can result in a higher antioxidant capacity. This suggests that it is more efficient to increase the extraction temperature instead of prolonging the extraction time.

Additionally, the antioxidant activity was measured in the rye bran residue after different extractions steps. The measurements have been performed using the QUENCHER procedure and the results are presented in **Table 3.8.** They indicate which extraction step is the most efficient in extracting the antioxidant compounds. After SC-CO_2 extraction, there has only been a slight increase in antioxidant activity; after removing all lipophilic components, the remaining non-lipophilic antioxidant concentration in the residue becomes higher and these components are usually responsible for these effects. PLE with acetone at high temperature (130 °C) is more effective than a longer extraction in lower temperatures according to all assays applied. The antioxidant activity in the residue remaining after acetone extraction at 70 °C is higher, thus the amount of valuable compounds remaining is larger. After a polar ethanol/water PLE in the $ABTS^{\bullet+}$ assay, a large decrease in antioxidant capacity (by half) in the residue has been determined. However, a similar decrease in TPC and $DPPH^{\bullet}$ in the other assays is only detected in the residue which remains after extraction at a lower temperature. When applying high temperature in the first step of PLE, the antioxidant activity in the residue decreases dramatically. If mild conditions are used, it might be possible to achieve a higher fractionation efficiency of valuable constituents.

Table 3.8. The antioxidant characteristics of solid substances obtained after SC-CO_2 extraction and PLE

Sample	TPC, mg GAE/g	$DPPH^{\bullet}$, mg TE/g	$ABTS^{\bullet+}$, mg TE/g	FRAP, mg TE/g
Before SC-CO_2	6.310 ± 0.182^{d}	12.393 ± 0.656^{d}	46.769 ± 5.624^{bc}	15.280 ± 0.848^{b}
After SC-CO_2	7.413 ± 0.128^{e}	13.395 ± 0.286^{e}	47.486 ± 2.165^{c}	14.550 ± 0.246^{b}
After ASE with acetone (130 °C, 40 min)	3.921 ± 0.258^{c}	6.837 ± 0.758^{b}	40.892 ± 0.657^{b}	12.209 ± 0.390^{a}
After ASE with acetone (70 °C, 80 min)	3.882 ± 0.069^{c}	10.543 ± 0.812^{c}	41.589 ± 4.259^{b}	16.467 ± 0.598^{c}
After ASE with ethanol/water (130 °C, 40 min)	3.000 ± 0.097^{b}	6.158 ± 0.106^{ab}	22.317 ± 2.268^{a}	n.d.
After ASE with ethano/water (70 °C, 80 min)	1.643 ± 0.248^{a}	5.471 ± 0.196^{a}	23.870 ± 0.234^{a}	n.d.

The values are presented as means ± standard deviations (n=3); the mean values followed by different letter are significantly different ($p<0.05$); n.a. – not determined

3.3. Biorefining of blackcurrant pomace into high value ingredients

Applying modern extraction and fractionation methods for biorefining of blackcurrant pomace into numerous products with specified composition and properties may markedly increase the effectiveness of its processing, mainly in developing high value components for human consumption. Subsequently, a multistep biorefining scheme for valorization of blackcurrant pomace in order to recover high value fractions has been developed. For this purpose, the SC-CO_2 extraction conditions have been optimized to obtain the highest lipophilic fraction, while combinations of PLE with EAE with different solvents have been applied to isolate other soluble substances (**Fig. 3.9**).

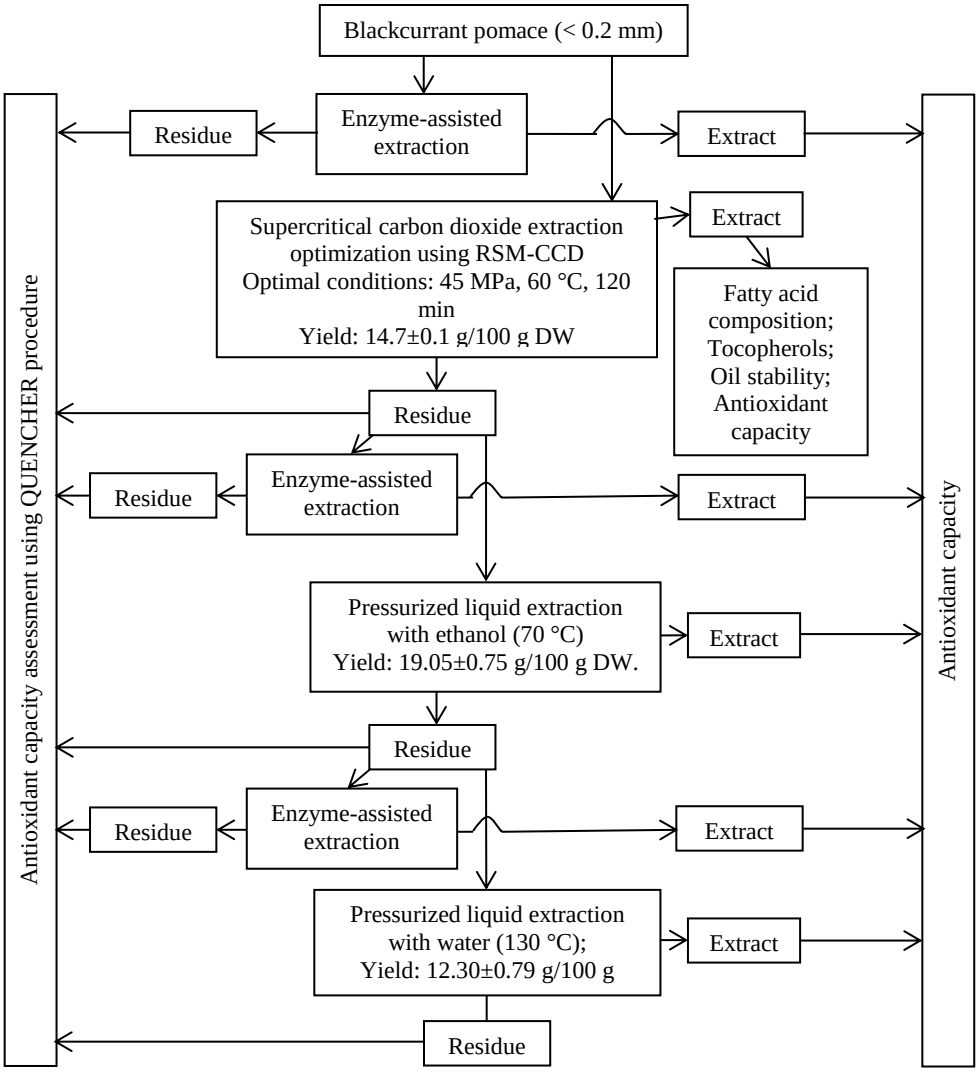


Figure 3.9. The biorefining scheme of blackcurrant pomace by SC-CO_2, PLE and EAE

3.3.1. The optimization of supercritical fluid extraction parameters

The main objective of the optimization of SC-CO_2 parameters is obtaining the maximal yield of extract. CCD was applied for selecting the set of parameters for extraction runs (**Table 3.9**), since several process parameters might be important for the extraction. Data shows that the differences between the yields obtained at the selected extraction parameters are not extensive (12.84−14.71 %); however, regression analysis of the data shows that SC-CO_2 yield is significantly (p<0.05) affected by the extraction pressure and temperature.

Response surface plots of the CCD for blackcurrant pomace are presented in **Figure 3.10** which shows the effect of independent variables on lipophilic extract yield. The graphs were obtained by keeping one of the variables constant (coded as zero level) while varying the other two variables and predicting the response. 3D graphs reveal the interactions between different parameters and evaluate their importance on the experimental response. At a constant extraction pressure (42.5 MPa) (**Fig. 3.10, *A*)**, the extract yield highly increases when the extraction temperature is raised. If the extraction temperature is increased from 30 to 60 °C, the yield grows from 13.5 to 14.4 g oil/100 g DW. Extending the extraction time has a minor influence on the extract recovery and prolonging the extraction time to over 120 min can be inefficient.

Rather interesting dependencies are noticeable when the temperature is fixed (**Fig. 3.10, *B***). The graph clearly shows which conditions produce the highest yield of extract. The yield of SC-CO_2 extract increases by 0.7 % when the extraction pressure is raised from 30 to 45 MPa. Further pressure increase results in a lower extraction rate. Again, as in the previous figure, the results of prolonging the extraction from 120 to 150 min are negligible. The most important parameters in the extraction optimization are temperature and pressure (**Fig. 3.10, *C***). The results of the research show that if both of these parameter values are raised, the yield of extract obtained from the extraction is higher. An increase in temperature from 30 to 60 °C results in 1 % gain of extract in the entire pressure range; however, increasing the pressure above 45 MPa does not result in a higher yield at the whole temperature range.

Table 3.9. The CCD parameter values and results of blackcurrant pomace extract optimization; 1–8 are experimental runs, 9–14 are axial point runs and 14–20 are central point runs

	Coded factor			Uncoded factor			
Run	Extraction pressure	Extraction temperature	Dynamic extraction time	Extraction pressure, MPa	Extraction temperature, °C	Dynamic extraction time, min	Yield, g/100 g DW
1	-1	-1	-1	30	30	60	12.90±0.01
2	1	-1	-1	55	30	60	13.26±0.37
3	-1	1	-1	30	60	60	14.17±0.32
4	1	1	-1	55	60	60	13.85±0.47
5	-1	-1	1	30	30	150	12.92±0.26
6	1	-1	1	55	30	150	13.62±0.27
7	-1	1	1	30	60	150	13.85±0.63
8	1	1	1	55	60	150	14.25±0.66
9	-1.682	0	0	21.48	45	105	12.84±0.47
10	1.682	0	0	63.52	45	105	13.42±1.20
11	0	-1.682	0	42.5	19.77	105	13.28±0.09
12	0	1.682	0	42.5	70.23	105	14.71±0.03
13	0	0	-1.682	42.5	45	29.32	13.32±1.20
14	0	0	1.682	42.5	45	180.68	13.69±1.03
15	0	0	0	42.5	45	105	13.78±0.36
16	0	0	0	42.5	45	105	13.60±0.67
17	0	0	0	42.5	45	105	14.03±0.27
18	0	0	0	42.5	45	105	13.90±0.20
19	0	0	0	42.5	45	105	14.04±0.09
20	0	0	0	42.5	45	105	14.14±0.56

Results are expressed as mean ± standard deviation of triplicate determinations.

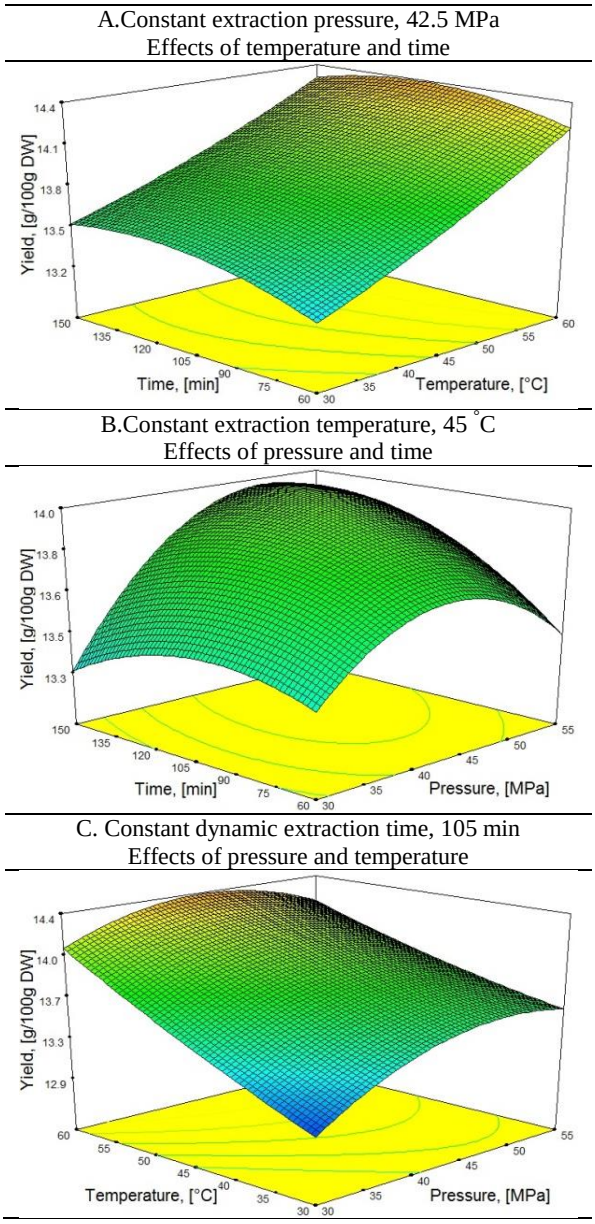


Figure 3.10. Response surface plots the effects of SC-CO_2 on the extract yield of blackcurrant pomace

The optimal SC-CO_2 extraction conditions for maximum extract yield (14.7±0.1 g/100 g DW) obtained by using CCD and RSM are 45 MPa pressure, 60 °C temperature and 120 min extraction time. For comparison, the yield of Soxhlet extraction with hexane is 11.44±0.26 g/100 g DW. A similar yield of extract (14.6 g/100 g DW) has been obtained in another study using SC-CO_2 for raspberry pomace (Kryzeviciute et al., 2016). Previously reported oil yield from *R. nigrum*

seeds ranges from 27 to 33 % (Bakowska-Barczak, Schieber, & Kolodziejczyk, 2009); however, this research only uses seeds for extraction with petroleum ether, while our study uses the whole pomace. To the best of our knowledge, only two studies have applied SC-CO_2 for blackcurrant pomace fractionation. In one of them, the residue after ethanol extraction was re-extracted by SC-CO_2 yielding <1 % of extract (Sandell et al., 2009). In the other, the seeds which were mechanically separated from the dried-press residue by wind-screening were extracted at 50 °C, 28−36 MPa for 270 min; however, the extract yield was not reported (Roj et al., 2009). The optimal conditions for the highest extract yield were tested using a bigger extraction system with a 500 mL extraction cell also testing the kinetics of the extraction (**Fig. 3.11**). The analysis shows that the majority of the extract is obtained during the first 60 min of extraction; therefore the effect of time in the selected range up to 180 min is not significant in the applied model.

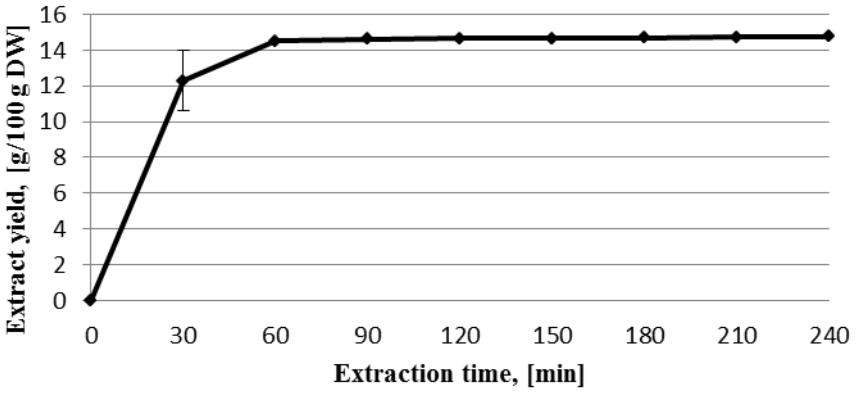


Figure 3.11. The kinetics of blackcurrant pomace SC-CO_2 extraction at optimal extraction parameters

3.3.2 The analysis of blackcurrant pomace supercritical fluid extraction optimization model

This study applies RSM with CCD to test three independent variables: pressure, temperature and dynamic extraction time. The ANOVA results are presented in **Table 3.10**. The calculated *F*-value (18.95) shows that our model is significant according to Student test (p<0.05). The lack of fit is not significant relative to pure error (p=0.9201). The determination coefficient is R^2=0.95 indicating a reasonable fit of the model; the actual and predicted model values are presented in **Figure 3.12.** An adequate precision of 16.809 is greater than 4 which indicate that the model can be used to navigate the design space. The model analysis shows that there are five significant model factors: P, T, Pt, P^2 and t^2. They can be arranged in order by their importance: temperature > pressure > time. The results of CCD analysis are expressed with the following second-order polynomial model equation (9):

$$Yield, \% = 13.917 + 0.155 \cdot P + 0.425 \cdot T + 0.079 \cdot t - 0.123 \cdot P \cdot t + 0.132 \cdot T \cdot t - 0.036 \cdot T \cdot t - 0.262 \cdot P^2 + 0.044 \cdot T^2 - 0.129 \cdot t^2 \quad (9)$$

Table 3.10. ANOVA for evaluated response surface quadratic model for blackcurrant pomace

Source	Sum of squares	df	Mean squares	*F*-ratio	*p*-value
Model	4.39	9	0.49	18.95	<0.0001*
P: Pressure	0.33	1	0.33	12.70	0.0051*
T: Temprature	2.47	1	2.47	96.09	<0.0001*
t: Time	0.09	1	0.09	3.31	0.0986**
PT	0.12	1	0.12	4.67	0.0561**
Pt	0.14	1	0.14	5.38	0.0428*
Tt	0.01	1	0.01	0.41	0.5365**
P^2	0.99	1	0.99	38.34	0.0001*
T^2	0.03	1	0.03	1.10	0.3181**
t^2	0.24	1	0.24	9.36	0.0121*
Residual	0.26	10	0.026		
Lack of fit	0.052	5	0.010	0.26	0.9201**
Pure error	0.21	5	0.011		
Cor Total	4.64	19			

* − significant values; ** − non-significant values

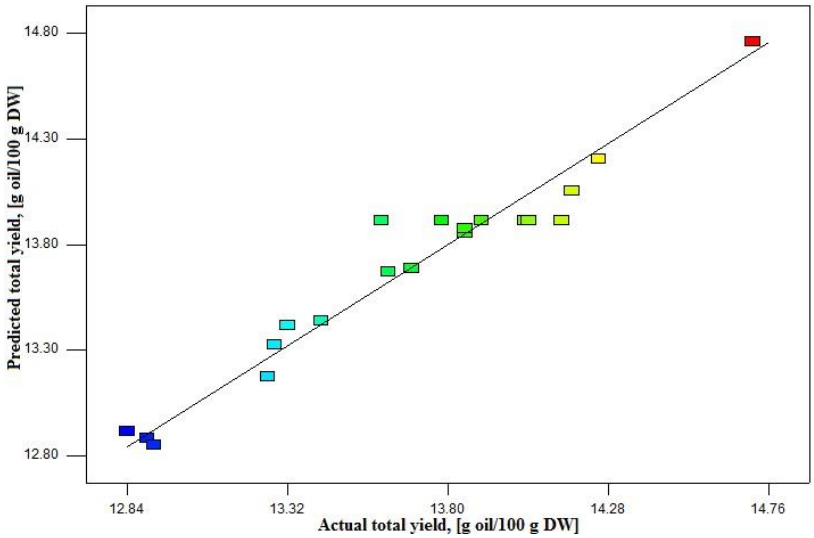


Figure 3.12. A comparison of actual and predicted extract yield from SC-CO_2 extraction of blackcurrant pomace (RSD values did not exceed 10 %)

3.3.3. Pressurized liquid and enzyme-assisted extraction of pomace

SC-CO_2 experiments reveal that this method may give up to 15 % of lipophilic extract from blackcurrant pomace. The extract obtained is of yellow greenish oil, while the residue after SC-CO_2 retains the dark color of blackcurrant indicating that natural pigments are not extracted with nonpolar CO_2 (**Fig. 3.13**). Next steps in biorefining are traditional extractions with increasing polarity solvents, acetone and EtOH/H_2O, or PLE with high polarity solvent ethanol with enzymatic treatment. The yields of acetone and EtOH/H_2O extracts are 17.50±0.78 % and 18.53±0.88%,

respectively; PLE-EtOH and PLE-H_2O enabled to recover 19.05±0.75 % and 12.30±0.79 % of extract from pomace residue after SC-CO_2. Moreover, Soxhlet and PLE extractions with EtOH provide similar yields; however, the extraction process in PLE is 16 times faster.

SC-CO_2 extract	Blackcurrant pomace			
	Raw (before extraction)	After SC-CO_2 at optimal conditions	After PLE with EtOH at 70 °C	After PLE with H_2O at 130 °C
				

Figure 3.13. The blackcurrant extract and residues obtained from different extractions

Conventional and high pressure extractions with different polarity solvents enable to isolate up to 50 % of soluble substances from blackcurrant pomace. Further attempts to increase the yield of soluble substances focused on enzymatic treatment. The process parameters were optimized by using RSM (data not showed); the following optimal parameters have been established for Viscozyme L: the ratio of enzyme with solids, E/S 6 % v/w (corresponds to 72 Fungal β-glucanase units (FBGU) for 10 g pomace); temperature of 40 °C; time – 7 hours; pH – 3.5. In addition, Celustar XL enzyme was used for comparison. The pomace was treated with enzymes before and after SC-CO_2 extraction and after PLE-EtOH. Provided that part of cell-wall bound water-soluble constituents should be extracted from pomace using water under pressurized conditions, residue after PLE-H_2O were not further utilized for EAE experiments. As given in **Table 3.12**, the highest yield of soluble substances is obtained from the pomace before and after SC-CO_2 extraction; however, the highest, double increase in the yield has been obtained from the pomace treated with Viscozyme after PLE-EtOH. CeluStar XL enzyme has been substantially less effective.

Table 3.11. Yields of soluble fractions of blackcurrant pomace after enzymatic treatment; enzyme/substrate ratio 6 % v/w, 40 °C, pH 3.5, 7 hours

Pomace	Yield*, g/100 g	Increase in soluble substance mass**	
		g/100 g	%
Before SC-CO_2 (Viscozyme L)	39.92±0.26[c]	17.97	94.89
After SC-CO_2 (Viscozyme L)	39.47±0.11[c]	18.28	86.27
After PLE-EtOH (Viscozyme L)	31.55± 0.07[b]	21.32	208.52
After PLE-EtOH (CeluStar XL)	16.98 ± 0.14[a]	6.76	66.06

* the yield was calculated excluding the weight of the freeze-dried supernatant form the appropriate control sample Blank B (enzyme+buffer); ** is calculated as the yield difference between freeze-dried supernatant of enzyme-treated sample and appropriate control sample Blank A (pomace+buffer), excluding the weight of the supernatant from appropriate control sample Blank C (buffer). The mean values followed by different letters are significantly different (p<0.05) in the column.

Consequently, the 3-step process, namely SC-CO_2, PLE-EtOH and treatment with Viscozyme provide approximately 67 % of extractable substances. Previously, EAE of antioxidative phenols was applied directly to the freeze-dried blackcurrant juice press residue and it demonstrated that various applied enzymes significantly increased plant cell-wall breakdown of the pomace as well as the availability of phenols for subsequent methanol extraction (Kapasakalidis, Rastall, & Gordon, 2009; Landbo & Meyer, 2001).

3.3.4. The analysis of fatty acid, tocopherols and oil stability of extract

CO_2 as an unpolar solvent is the most effective for the isolation of lipophilic compounds. In general, SC-CO_2 extraction of seeds or plant material containing considerable amounts of seeds, as berry pomace, gives oily extracts. Therefore, the composition of lipid substances determines the value of such extracts. Triacylglycerols are the main components of oily extracts and their nutritional value depends on the fatty acid composition. In the present study, the FA composition of blackcurrant pomace extracts obtained by SC-CO_2 and Soxhlet was analyzed by GC-FID and compared with the previously published data (**Table 3.12**). Research shows that FA composition is similar in blackcurrant pomace oil obtained by different extraction techniques: the dominant FA is linoleic (46.89−47.21 %), γ-linolenic (14.02−14.08 %), linolenic (13.80−13.88 %) and oleic (11.84−11.79 %). Similar FA composition was reported by Yang (Yang et al., 2011) in the oil isolated from seeds separated by SC-CO_2, while the compositions of FA in the oils isolated with organic solvents were slightly different (**Table 3.12**). For instance, the oil analyzed by Pieszka (Pieszka et al., 2015) contained twice as much of palmitic acid, although the percentage of unsaturated acids was lower, except for α-linolenic acid. Among the minor FA's, a significant amount of behenic acid was reported in the aforementioned article.

The composition of FA may depend on plant cultivar (Bakowska-Barczak et al., 2009); for instance the contents of γ- and α-linolenic acids in 29 blackcurrant

genotypes are in the ranges of 11.6−22.7 % and 11.1−18.7 % (Castillo et al., 2004). Unsaturated FA's (linoleic, linolenic, and oleic) compose the highest content (~86 %) of the total amount in SC-CO_2 extract of blackcurrant pomace. Considering that health claims for α-linolenic and linoleic acids (both contribute to the maintenance of normal blood cholesterol levels) have been approved in Commission Regulation no. 432 (EU, 2012), SC-CO_2 extract of blackcurrants may be considered as a valuable ingredient for the formulation of food supplements and other relevant products.

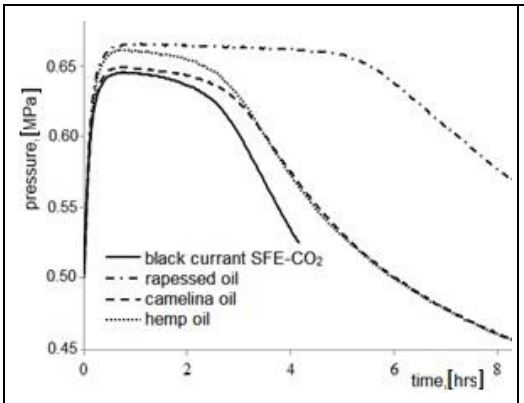
Table 3.12. The composition of fatty acids (g/100 g) in pomace oil extracted by SC-CO_2 and Soxhlet

Fatty Acid	SC-CO_2	*n*-hexane	(Yang et al., 2011)	(Pieszka et al., 2015)	(Bakowska-Barczak et al., 2009)
Myristic (C14:0)	0.07	0.06	-	0.47	tr−0.1
Palmitic (C16:0)	6.41	6.02	5.7	12.11	5.8−6.6
Palmitoleic (C16:1)	0.10	0.08	-	0.36	0.2−0.3
Heptadecanoic (C17:0)	0.06	0.05	-	-	-
Stearic (C18:0)	1.52	1.50	1.5	1.66	1.5−1.9
Oleic (C18:1)	11.84	11.79	13.4	10.47	11.0−12.3
Linolelaidic (C18:2)	0.11	0.11	0.7	-	-
Linoleic (C18:2)	46.89	47.21	46.1	41.56	43.9−47.5
Arachidic (C20:0)	0.27	0.27	-	2.37	2.37
γ-Linolenic (C18:3n6)	14.02	14.08	15.0	8.01	10.9−16.7
cis-11-Eicosenoic (C20:1)	0.86	0.93	1.0	0.76	1.0−1.4
Linolenic (C18:3)	13.80	13.88	13.6	15.17	14.1−18.1
cis-11,14-Eicosadienoic (C20:2)	3.02	3.05	-	0.38	0.3
Behenic (C22:0)	0.14	0.16	-	2.55	0.1
cis-11,14,17-Eicosatrienoic (C20:3)	0.03	0.04	-	-	-
Lignoceric (C24:0)	0.54	0.13	-	-	tr−0.1

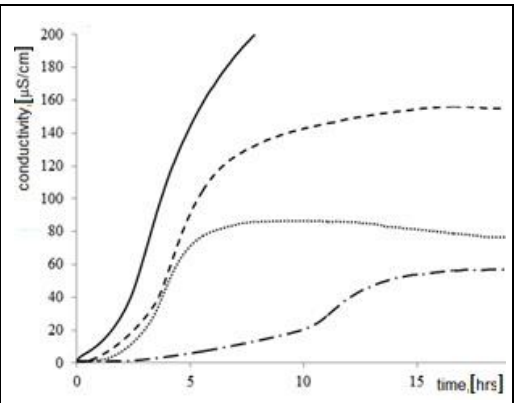
*Values are averages of two replicates; the variation coefficient of the method is below 3 %.

Tocopherols are the most important lipophilic antioxidants in biological systems. Their concentrations in SC-CO_2 extract isolated at optimal conditions are as follows (in μg/g oil): α-tocopherol – 789±33; γ-tocopherol – 1511±27; δ-tocopherol – 168±4; total – 2468. The composition of tocopherols in previously studied blackcurrant seed oil isolated by SC-CO_2 was different (in mg/g oil): α-tocopherol – 0.1; γ-tocopherol – 0.9; δ-tocopherol – 0.1 (Yang et al., 2011). It appears that the content of tocopherols in *R. nigrum* seeds depends on the plant cultivar; it may vary from 1.228 to 2.458 mg/g oil among 10 studied breeds (Goffman & Galletti, 2001). Comparing with the reported data, the SC-CO_2 extract obtained in our study is rich in Vitamin E, which, according to the approved health claims, protects the cells from oxidative stress (EU, 2012).

The peroxide value of freshly extracted blackcurrant pomace oil is 2.43±0.09 meq/kg. Its oxidative oil stability was compared with rapeseed, camelina and hemp oils with the peroxide values of 2.93, 2.02 and 1.76 meq/kg, respectively (**Fig. 3.14**). The analysis shows that blackcurrant SC-CO_2 oil is rapidly oxidizing during Oxipres and Rancimat assays. The induction periods for blackcurrant oil are 2.39 and 2.46 h, respectively. These values for rapeseed, camelina and hemp oils were 5.37 and 10.48 and 2.78, and 3.45, 2.67 and 3.42 h, respectively. Therefore, blackcurrant oil consisting mainly of unstable polyunsaturated acids should be carefully handled, i.e. avoiding contact with oxygen, protecting it against direct light and working at lower temperatures. Antioxidant additives would most likely be necessary to prolong the shelf-life of the extract.



A: Oxipres method



B: Rancimat method

Figure 3.14. The oxidation curves of blackcurrant pomace oil isolated by SC-CO_2 and selected commercial oils

3.3.5. TPC and antioxidant activity of extracts and solid residues

TPC of extracts obtained by two different extraction methods, Soxhlet and SC-CO_2, with nonpolar and low polarity solvents show no significant difference, as **Table 3.13** shows. TPC of EtOH/H_2O extract is more than 4 times higher than in other extracts, indicating that polar antioxidatively active compounds are dominant in blackcurrant pomace. The content of extractable TPC does not change after SC-CO_2 but it is considerably lower when using the pomace residue of PLE-EtOH. It indicates that ethanol, as a polar solvent, is effective in extracting polyphenolics present in blackcurrant pomace. Other antioxidant activity indicators of pomace extracts obtained by CO_2 and hexane are somewhat similar; however, acetone and EtOH/H_2O extracts are much stronger antioxidants compared to lipid extracts. For instance, ORAC and $TEAC_{ABTS}$ values of EtOH/H_2O extracts are 661.9 and 612.0 mg TE/g.

Table 3.13. Total phenolic content and the antioxidant properties of extracts from Soxhlet extraction, SC-CO_2 and PLE

Extracts and solid substances	TPC, mg GAE/g	DPPH, mg TE/g	ORAC, g TE/g	FRAP, mg TE/g
Extracts				
Hexane	27.02±1.57[a]	2.31±0.20[a]	13.24±0.92[a]	25.23±2.21[a]
Acetone	27.49±1.43[a]	40.69±3.43[b]	261.4±21.6[b]	70.80±2.62[b]
Ethanol/water	119.5±6.5[d]	172.1±10.6[c]	661.9±48.9[d]	348.6±23.0[c]
SC-CO_2	24.34±1.45[a]	1.59±0.08[a]	11.35±1.15[a]	25.00±0.97[a]
PLE-EtOH	64.75±0.94[b]	n.a.	330.6±18.6[c]	n.a.
PLE-H_2O	79.84±0.70[c]	n.a.	329.3±16.4[c]	n.a.
Pomace and residues after extractions				
Raw freeze dried	23.60±1.56[d]	54.80±4.76[b]	51.81±4.60[c]	106.4±7.2[b]
After hexane	27.12±1.66[e]	72.69±8.06[c]	91.29±7.19[e]	121.3±5.8[bc]
After acetone	22.05±1.46[d]	78.46±5.76[c]	56.80±3.62[c]	135.9±10.6[c]
After EtOH/H_2O	10.07±0.41[b]	14.45±0.18[a]	5.67±0.36[a]	57.02±3.40[a]
After SC-CO_2	27.83±1.62[e]	77.34±6.61[c]	79.19±3.58[d]	114.4±9.8[b]
After PLE-EtOH	16.71±0.53[c]	n.a.	39.71±2.12[b]	n.a.
After PLE-H_2O	3.80±0.17[a]	n.a.	5.51±0.73[e]	n.a.

* n.a. = not applied. The mean values followed by different letter are significantly different (p<0.05) in the column.

TPC values of the solid pomace residue were also measured using the so-called QUENCHER procedure (**Tables 3.13, 3.15**). This procedure enables measuring all antioxidatively active groups, i.e. the free ones which are accessible for extraction, and the bound ones which cannot be extracted. The results obtained by this method provide additional important information about the efficiency of biorefining. After applying acetone, TPC in the pomace decreases only slightly, while the highest decrease (by more than half) in TPC has been determined in the residue after extraction with EtOH/H_2O. PLE-EtOH also considerably decreases TPC in the untreated pomace; however the EtOH/H_2O extraction residue presents a lower value of TPC than the residue of PLE-EtOH. This indicates that water is an efficient co-solvent for isolating polyphenolics from the pomace. Higher extract yields and antioxidant activities were reported in fractioned high pressure extraction of anthocyanins from elderberry pomace when water was present, both in the raw material and in the solvent mixture (Seabra et al., 2010). After SC-CO_2 and hexane extractions, all other antioxidant capacity values in the pomace residue increase, which is in agreement with TPC values. After acetone extraction, the ORAC value considerably decreases, while DPPH$^{\bullet}$ scavenging and FRAP values slightly increase. All antioxidant capacity values decrease substantially after EtOH/H_2O extraction; for instance, the ORAC value is 10 times lower.

Antioxidant activity values in EAE were measured both for extracts and solid residues (**Tables 3.14, 3.15**). In addition, AOA was calculated for 1 g of pomace.

Table 3.14. The antioxidant characteristics of soluble substances (extracts) obtained after EAE of blackcurrant pomace and corresponding control samples prior and after SC-CO_2, and after PLE-EtOH

Pomace samples	TPC		$ABTS^{\bullet+}$		ORAC	
	mg GAE/g extract	mg GAE/g pomace*	mg GAE/g extract	mg GAE/g pomace*	mg GAE/g extract	mg GAE/g pomace*
Before SC-CO_2:						
Pomace + Viscozyme L	21.11±0.49^{b}	8.43±0.20^{e}	107.90±2.40^{b}	43.09±0.98^{e}	60.90±1.90^{b}	24.31±0.76^{d}
Pomace + Buffer (*Blank A*)	36.32±0.60^{e}	7.97±0.13^{d}	183.00±2.90^{e}	40.16±0.65^{d}	117.70±6.00^{e}	25.84±1.31^{d}
After SC-CO_2:						
Pomace + Viscozyme L	21.14±0.76^{b}	8.34±0.30^{e}	109.90±3.10^{b}	43.38±1.22^{e}	79.74±8.35^{c}	31.47±3.30^{e}
Pomace + Buffer (*Blank A*)	33.23±0.75^{d}	7.04±0.16^{c}	162.70±3.00^{d}	34.47±0.64^{c}	95.21±3.88^{d}	20.17±0.82^{c}
After PLE-EtOH:						
Pomace + Viscozyme L	9.29±0.60^{a}	2.93±0.19^{a}	54.78±3.18^{a}	17.28±1.00^{b}	18.86±1.33^{a}	5.95±0.42^{a}
Pomace + CeluStar XL	20.86 ± 0.31^{b}	3.54 ± 0.05^{b}	109.50 ± 6.10^{b}	18.60±1.04^{b}	64.31±5.78^{b}	10.92±0.98^{b}
Pomace + Buffer (*Blank A*)	26.43±0.58^{c}	2.70±0.06^{a}	132.20 ± 2.80^{c}	13.52±0.29^{a}	91.32±7.14^{d}	9.34±0.73^{b}

* The contribution of EAE-derived extract to the total activity of pomace before SC-CO_2 and pomace residues after SC-CO_2 and PLE-EtOH was calculated taking into account the yield of the appropriate supernatant (**Table 3.11**). The mean values followed by different letters are significantly different (p <0.05) in the column.

The research shows that AOA values are considerably higher in extracts untreated with enzymes; however the values expressed in 1 g of pomace are slightly higher when the pomace is treated with enzymes. It can be explained by the fact that the yields of soluble substances in EAE are notably higher as compared to the extraction with a buffer (**Table 3.11**). These findings also indicate that enzymatic treatment may assist in releasing phenolic and radical scavenging compounds.

A slight increase in AOA has been detected after SC-CO_2 extraction, while after PLE-EtOH extraction has decreased it significantly in all of the applied methods. Celustar XL enzyme (xylanase enzyme) extracts show higher antioxidant activity capacity but the extract yield is 2 times lower than using Viscozyme L (cellulotic enzyme mixture). The TPC values in the residue after EAE (using a buffer without enzymes) increase slightly after SC-CO_2 extraction, while the main decline is detected after PLE-EtOH (**Table 3.15**). Treating the pomace with a buffer before PLE-EtOH reduces the TPC to a lesser extent, while using Viscozyme L produces similar results to the pomace after SC-CO_2 extraction. EAE of PLE-EtOH pomace residue produces three times as much of extract; however, the TPC extracted from 1 g of pomace is only slightly higher as compared to extraction with a buffer. Consequently, the amount of residue in EAE of PLE-EtOH residue is radically lower and the concentration of TPC remains unchanged after EAE.

Table 3.15. The effects of enzymes on the antioxidant characteristics of solid substances obtained after EAE of blackcurrant pomace and corresponding control samples prior and after SC-CO_2, and after PLE-EtOH

Pomace samples	TPC, mg GAE/g pomace	$ABTS^{\bullet+}$, mg TE/g pomace	ORAC, mg TE/g pomace
Before SC-CO_2:			
Untreated pomace	23.52 ± 1.56^{bc}	261.20 ± 20.90^{c}	65.35 ± 1.96^{f}
Pomace + Viscozyme L	21.20 ± 1.98^{b}	205.80 ± 8.00^{b}	46.87 ± 1.68^{de}
Pomace + Buffer (*Blank A*)	25.24 ± 1.64^{bc}	202.60 ± 15.60^{b}	41.39 ± 2.77^{d}
After SC-CO_2:			
Untreated pomace	26.87 ± 1.01^{c}	234.90 ± 4.60^{bc}	77.87 ± 5.86^{g}
Pomace + Viscozyme L	25.97 ± 2.23^{c}	219.50 ± 21.30^{bc}	41.00 ± 3.18^{d}
Pomace + Buffer (*Blank A*)	25.76 ± 1.83^{c}	263.30 ± 25.40^{c}	53.48 ± 3.22^{e}
After PLE-EtOH:			
Untreated pomace	16.71 ± 0.53^{a}	152.40 ± 15.80^{a}	39.71 ± 2.12^{cd}
Pomace + Viscozyme L	26.25 ± 2.57^{c}	336.20 ± 26.00^{d}	27.71 ± 2.27^{ab}
Pomace + CeluStar XL	22.86 ± 1.10^{bc}	254.20 ± 19.40^{bc}	22.98 ± 0.85^{a}
Pomace + Buffer (*Blank A*)	21.20 ± 1.55^{b}	262.80 ± 24.90^{c}	33.14 ± 3.12^{bc}

The mean values followed by different letter are significantly different ($p<0.05$) in the column.

TPC in the residue after EtOH/H_2O extraction is 10.07 mg GAE/g and after EAE of PLE-EtOH this value is 26.25 mg GAE/g; the TPC value in the residue without enzymatic treatment is slightly lower (16.71 mg GAE/g). Applying EAE produces an extract which is rich in various bioactive compounds and the residue of the extraction contains high concentrations of TPC and other AOA acting substances. It was recently reported that polyphenol-rich blackcurrant extract may prevent metabolic dysfunctions induced by diets high in fat and cholesterol, according to research in mice (Benn et al., 2014).

CONCLUSIONS

1. The yields obtained with pressurized liquid extraction and methanol/water (extracting polar compounds) from rye and wheat brans are considerably higher in comparison to the extract yields isolated with hexane and acetone. In general, the accumulative yields of extracts isolated from rye bran are higher in comparison to wheat bran. The yield of methanol extract of wheat and rye bran can be increased by decreasing the particle size.
2. Rye bran extracts, in general, are stronger antioxidants than wheat bran extracts. The amounts of total phenolic content in rye bran extract isolated with methanol (0.83−1.42 mg GAE/g DW) are about 2 times higher than in hexane extract (0.31−0.68 GAE/g DW) and 3 times higher than in extract isolated using acetone (0.33−0.48 mg GAE/g DW). Radical scavenging capacity of $ABTS^{\bullet+}$ assay reveals notable differences between rye and wheat brans with regards to decreasing the particle size: in rye bran it decreases (from 21.45 to 12.25 μM TE/g DW) and in wheat bran it increases (from 9.72 to 17.44 μM TE/g DW).
3. Supercritical fluid extraction conditions have been optimized and the highest rye bran oil yield (2.49±0.01 g oil/100 g DW) is obtained at 55 MPa pressure and 70 °C temperature in 120 min of extraction. It is established that in terms of significance these parameters can be arranged in the following order: temperature > pressure > dynamic extraction time. The extract of rye bran oil appears to be rich in unsaturated fatty acids (up to 82 g/100 g oil).
4. The highest yield for rye bran residue (after supercritical fluid extraction) using pressurized liquid extraction with acetone can be obtained using 130 °C temperature for 41 min of extraction – 1.96±0.11 g/100 g DW. The extract yield using pressurized liquid extraction system with ethanol/water mixture is 16.93±0.48 g/100 g DW (at 70°C and 81 min) and 16.86±1.21 g/100 g DW (at 130 °C and 41 min). Pressurized liquid extraction using ethanol/water mixture appears to be the most efficient for extracting phenolic antioxidant compounds from the rye bran residue and the amount of total phenolic compounds in the final residue is small (1.643±0.248 mg GAE/g).
5. Applying the scheme developed for biorefining blackcurrant pomace using high pressure and enzyme-assisted extraction 3 valuable fractions can be obtained: supercritical carbon dioxide extraction at optimized conditions (45 MPa pressure, 60 °C temperature, 120 min extraction time) gives 14.7±0.1 g/100 g DW extract yield; pressurized liquid extraction of the residue (after supercritical fluid extraction) with ethanol (19.05±0.75 g/100g DW) and water (12.30±0.79 g/100 g DW) provide 2 more fractions. Additional enzyme-assisted extraction step can considerably increase the extraction yield of soluble substances (from 50 up to 67 g/100 g DW).
6. The products obtained by biorefining of blackcurrant pomace using high pressure and enzyme-assisted extractions may be considered as valuable ingredients for producing food supplements and functional foods. Lipophilic extracts may be used for polyunsaturated fatty acids (up to 86 g/100 g oil) or for vitamin E (up to 2.47 mg/g EDW) supplement production. Ethanol (64.75±0.94 mg GAE/g EDW)

and water (79.84±0.70 mg GAE/g EDW), as polar solvents, seem to be effective in extracting polyphenolics present in blackcurrant pomace. Further enzymatic treatment may assist in releasing phenolic and radical scavenging compounds.

REFERENCES

1. ABOZED, S. S., El-kalyoubi, M., Abdelrashid, A., Salama, M.F. Total phenolic contents and antioxidant activities of various solvent extracts from whole wheat and bran. *Annals of Agricultural Sciences*. 2014, 59(1), 63-67. ISSN 0570-1783.

2. AGIL, R., Oomah, D. B., Mazza, G., Hosseinian, F. S. Optimization of alkylresorcinols extraction from triticale bran using response surface methodology. *Food and Bioprocess Technology*. 2012 5(7), 2655–2664. ISSN 1935-5130.

3. AKAY, F., Kazan, A., Celiktas, M. S., Yesil-Celiktas, O. A holistic engineering approach for utilization of olive pomace. *Journal of Supercritical Fluids*. 2015, 99, 1–7. ISSN 0896-8446

4. ANDERSON, M. J., & Whitcomb, P. J. *DOE Simplified. Practical Tools for Effective Experimentation. Third Edition*. CRC Press, Taylor and Francis Group, p. 268 ISBN 9781482218947, 2016.

5. ANDERSSON, A. A. M., Dimberg, L., Åman, P., & Landberg, R. Recent findings on certain bioactive components in whole grain wheat and rye. *Journal of Cereal Science*. 2014, 59(3), 294–311. ISSN 0733-5210.

6. ANDREASEN, M. F., Landbo, A. K., Christensen, L. P., Hansen, A., & Meyer, A. S. Antioxidant effects of phenolic rye (Secale cereale L.) extracts, monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low-density lipoproteins. *Journal of Agricultural and Food Chemistry*. 2001, 49(8), 4090–4096. ISSN 0021-8561.

7. ANTOLOVICH, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. Methods for testing antioxidant activity. *The Analyst*. 2002, 127(1), 183–198. ISSN 0003-2654.

8. ANTTONEN, M. J., & Karjalainen, R. O. High-performance liquid chromatography analysis of black currant (Ribes nigrum L.) Fruit phenolics grown either conventionally or organically. *Journal of Agricultural and Food Chemistry*. 2006, 54, 7530–7538. ISSN 0021-8561.

9. ANWAR, F., Abdul Qayyum, H. M., Ijaz Hussain, A., & Iqbal, S. Antioxidant activity of 100% and 80% methanol extracts from barley seeds (*Hordeum vulgare* L.): stabilization of sunflower oil. *Grasas Y Aceites*. 2010, 61(3), 237–243. ISSN 0017-3495.

10. APPRICH, S., Tirpanalan, Ö., Hell, J., Reisinger, M., Böhmdorfer, S., Siebenhandl-Ehn, S., Kneifel, W. Wheat bran-based biorefinery 2: Valorization of products. *LWT - Food Science and Technology*. 2014, 56(2), 222-231. ISSN 0023-6438

11. ARAUJO, P. W., & Brereton, G. (1996). Experimental design. I. Screening. *TrAC - Trends in Analytical ChemistrY*. 1996, 15(1), 26–31. ISSN 0165-9936.

12. ATHUKORALA, Y., Hosseinian, F. S., & Mazza, G. Extraction and fractionation of alkylresorcinols from triticale bran by two-step supercritical carbon dioxide. *LWT - Food Science and Technology*. 2010, 43(4), 660–665. ISSN 0023-6438.

13. AZMIR, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Omar, a. K. M. Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering*. 2013, 117(4), 426–436. ISSN 0260-8774.

14. BAKOWSKA-BARCZAK, A. M., Schieber, A., & Kolodziejczyk, P. Characterization of Canadian black currant (Ribes nigrum L.) seed oils and residues. *Journal of Agricultural and Food Chemistry*. 2009, 57(24), 11528–11536. ISSN 0021-8561.

15. BARANAUSKIENĖ, R., Kazernavičiute, R., Pukalskiene, M., Maždžieriene, R., & Venskutonis, P. R. (2014). Agrorefinery of Tanacetum vulgare L. into valuable products and evaluation of their antioxidant properties and phytochemical composition. *Industrial Crops and Products*. 2014, 60, 113–122. ISSN 0926-6690.

16. BARTLOVA, M., Bernášek, P., Sýkora, J., & Sovová, H. HPLC in reversed phase mode: Tool for investigation of kinetics of blackcurrant seed oil lipolysis in supercritical carbon dioxide. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. 2006, 839(1-2), 80–84. ISSN 1570-0232.

17. BARTOSZ, G. *Food oxidants and Antioxidants. Chemical, Biological and Functional Properties.* CRC Press. Taylor and Francis group. 2014, p. 568. ISBN 9781439882412

18. BASS, E. J. Wheat flour milling. *Wheat: Chemistry and Technology*, 1988, *2*, 1–68.

19. BENN, T., Kim, B., Park, Y. K., Wegner, C. J., Harness, E., Nam, T. G., Lee, J. Y. Polyphenol-rich blackcurrant extract prevents inflammation in diet-induced obese mice. *Journal of Nutritional Biochemistry*. 2014, 25(10), 1019–1025. ISSN 0955-2863.

20. BENZIE, I. F., & Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*. 1996, 239(1), 70–6. ISSN 0003-2697.

21. BEZZERA, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*. 2008, 76(5), 965–977. ISSN 0039-9140.

22. BISHAYEE, A., Mbimba, T., Thoppil, R. J., Haznagy-Radnai, E., Sipos, P., Darvesh, A. S., Hohmann, J. Anthocyanin-rich black currant (Ribes nigrum L.) extract affords chemoprevention against diethylnitrosamine-induced hepatocellular carcinogenesis in rats. *Journal of Nutritional Biochemistry*. 2011, 22(11), 1035–1046. ISSN 0955-2863.

23. BLEVE, M., Ciurlia, L., Erroi, E., Lionetto, G., Longo, L., Rescio, L., Vasapollo, G. An innovative method for the purification of anthocyanins from grape skin extracts by using liquid and sub-critical carbon dioxide. *Separation and Purification Technology*. 2008, 64(2), 192–197. ISSN 1383-5866.

24. BOX, G. E. P., & Wilson, K. B. On the experimental attainment of optimum conditions. *Journal of the Royal Statistical Society*. 1951, 13(1), 1–45.

25. BRAND-WILLIAMS, W., Cuvelier, M. E., & Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*. 1995, 28(1), 25–30. ISSN 0023-6438.

26. BREWER, L. R., Kubola, J., Siriamornpun, S., Herald, T. J., & Shi, Y. C. Wheat bran particle size influence on phytochemical extractability and antioxidant properties. *Food Chemistry*. 2014, 152, 483–490. ISSN 0308-8146.

27. BRILLOUET, J. M., & Joseleau, J. P. Investigation of the structure of a heteroxylan from the outer pericarp (beeswing bran) of wheat kernel. *Carbohydrate Research*. 1987, 159(1), 109–126. ISSN 0008-6215.

28. BURDICK, R. K., Borror, C. M., & Montgomery, D. C. *Design and analysis of gauge R&R studies: making decisions with confidence intervals in random and mixed ANOVA models*. *Quality*. 2005.

29. CAMPBELL, G., Koutinas, A., Wang, R., Sadhukhan, J., & Webb, C. Cereal potential. *Chemical Engineering*. 2006, 781, 26–28. ISSN 1385-8947.

30. CARLSEN, C. U., Møller, J. K. S., & Skibsted, L. H. Heme-iron in lipid oxidation. *Coordination Chemistry Reviews*. 2005, 249(3-4), 485-498. ISSN 0010-8545.

31. CAROCHO, M., & Ferreira, I. C. F. R. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology*. 2013, 51, 15-25. ISSN 0278-6915.

32. CHARLES, D. J. *Antioxidant properties of spices, herbs and other sources*. *Antioxidant Properties of Spices, Herbs and Other Sources*. p. 612, 2013. ISBN 978-1-4614-4310-0.

33. CHEMISTS, A. of O. A. Official methods of analysis of AOAC International. *Association of Official Analysis Chemists International*, 1995.

34. CHERUBINI, F. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management*. 2010, 51(7), 1412–1421. ISSN 0196-8904.

35. CHRZANOWSKI, G. Influence of phenolic acids isolated from blackcurrant and sour cherry leaves on grain aphid (Sitobion avenae F.). *Pestycydy*. 2008, (1-2), 127–133. ISSN 0208-8703.

36. DECKER, E. A., Elias, R. J., Julian McClements, D., Martín-Belloso, O., Odriozola-Serrano, I., Rojas-Graü, M. A., & Soliva-Fortuny, R. *Oxidation in Foods and Beverages and Antioxidant Applications*. *Oxidation in Foods and Beverages and Antioxidant Applications*. A volume in Woodhead Publishing Series in Food Science, Technology and Nutrition. 2010, p. 528. ISBN: 978-1-84569-983-3

37. DEY, E. S., & Mikhailopulo, K. A food grade approach for the isolation of major alkylresorcinols (ARs) from rye bran applying tailored supercritical carbon dioxide (scCO2) extraction combined with HPLC. *Journal of Supercritical Fluids*. 2009, 51(2), 167–173. ISSN 0896-8446.

38. DEY, E. S., Ahmadi-Afzadib, M., Nybomb, H., & Ibrahim, T. Alkylresorcinols isolated from rye bran by supercritical fluid of carbon dioxide and suspended in a food-grade emulsion show activity against Penicillium expansum on apples. *Archives of Phytopathology and Plant Protection*. 2013, 46(1), 105–119. ISSN 0323-5408.

39. DI LENA, G., Vivanti, V., & Quaglia, G. B. Amino acid composition of wheat milling by-products after bioconversion by edible fungi mycelia. *Nahrung - Food*. 1997, 41(5), 285–288. ISSN 1613-4133.

40. DOBSON, G., Shrestha, M., Hilz, H., Karjalainen, R., Mcdougall, G., & Stewart, D. Lipophilic components in black currant seed and pomace extracts. *European Journal of Lipid Science and Technology*. 2012, 114(5), 575–582. ISSN 1438-9312.

41. DURANTE, M., Lenucci, M. S., Rescio, L., Mita, G., & Caretto, S. Durum wheat by-products as natural sources of valuable nutrients. *Phytochemistry Reviews*. 2012, 11(2), 255-

262. ISSN 1568-7767.

42. DZIKI, D., & Laskowski, J. Wheat kernel physical properties and milling process. *Acta Agrophysica*. 2005, 6(1), 59–71. ISSN 2084-3429

43. ELLIOTT, D. C., Orth, R. J., Gao, J., Werpy, T. A., Eakin, D. E., Schmidt, A. J., Landucci, R. Biorefinery Concept Development Based on Wheat Flour Milling. *Fuel Chemistry Division Preprints*. 2002, 47(1), 361–362. ISSN 0140-6701.

44. EL-SHAMI, S. M., El-Mallah, M. H., Hassanein, M. M. M., & Abdel-Razek, A. G. Elucidation of Polynutrients and Fatty Acid Patterns of Wheat Germ Oil Extracted by. *Journal of Applied Sciences Research*. 2011, 7(12), 1840–1846. ISSN 1816-157X.

45. ESCUDERO, A. E., & Gonzalez, S. P. Dietary fibre. *Nutricion Hospitalaria*, *21 Suppl.* 2006, 2, 60–72. ISSN 0212-1611

46. European Commission. *Preparatory Study on Food Waste Across Eu 27*. *October* (Vol. 33). 2010.

47. European Food Safety Authority. Scientific opinion on dietary reference values for carbohydrates and dietary fibre. EFSA panel on dietetic products, nutrition, and allergies (nda). *EFSA Journal*. 2010, *8*(3), 1462–1538. ISSN 1831-4732.

48. FAO. (2014). FAO statistics. Retrieved from http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/E\nhttp://faostat3.fao.org/ [2016 05 20].

49. FIGUEROLA, F., Hurtado, M. L., Estavez, A. M., Chiffelle, I., & Asenjo, F. Fibre concentrates from apple pomace and citrus peel as potential fibre sources for food enrichment. *Food Chemistry*. 2005, 91(3), 395–401. ISSN 0308-8146.

50. FOLIN, O., & Ciocalteu, V. Tyrosine and tryptophan determinations proteins. *Journal of Biological Chemistry*. 1927, 73, 627–650. ISSN 1083-351X.

51. FRANCISCO, J. D. C., Danielsson, B., Kozubek, A., & Dey, E. S. Application of supercritical carbon dioxide for the extraction of alkylresorcinols from rye bran. *Journal of Supercritical Fluids*. 2005a, 35(3), 220–226. ISSN 0896-8446.

52. FRANCISCO, J. D. C., Danielsson, B., Kozubek, A., & Dey, E. S. Extraction of rye bran by supercritical carbon dioxide: Influence of temperature, CO_2, and cosolvent flow rates. *Journal of Agricultural and Food* Chemistry. 2005b, 53(19), 7432–7437. ISSN 0021-8561.

53. FRANKEL, E. *Lipid oxidation. Second Edition.* University of California, USA. 2005. ISBN 9780953194988.

54. GALANAKIS, C. M. Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. *Trends in Food Science and Technology*. 2012, 26(2), 68-87. ISSN 0924-2244.

55. GALANAKIS, C. M., Goulas, V., Tsakona, S., Manganaris, G. A., & Gekas, V. A Knowledge Base for the Recovery of Natural Phenols with Different Solvents. *International Journal of Food Properties*. 2011, 16(2), 382-396. ISSN 1094-2912.

56. GLITSO, L. V., & Bach Knudsen, K. E. Milling of Whole Grain Rye to Obtain Fractions with Different Dietary Fibre Characteristics. *Journal of Cereal Science*. 1999, 29(1), 89–97. ISSN 0733-5210

57. GOFFMAN, F. D., & Galletti, S. Gamma-linolenic acid and tocopherol contents in the seed oil of 47 accessions from several ribes species. *Journal of Agricultural and Food Chemistry*. 2001, 49(1), 349–354. ISSN 0021-8561.

58. GOKMEN, V., Serpen, A., & Fogliano, V. Direct measurement of the total antioxidant capacity of foods: the "QUENCHER" approach. *Trends in Food Science & Technology*. 2009, 20(6-7), 278–288. ISSN 0924-2244.

59. GOPLAN, A., Reuben, S. C., Ahmed, S., Darvesh, A. S., Hohmann, J., & Bishayee, A. The health benefits of blackcurrants. *Food & Function*. 2012, 3(8), 795-809. ISSN 2042-6496

60. GRUSZKA, J., & Kruk, J. RP-LC for determination of plastochromanol, tocotrienols and tocopherols in plant oils. *Chromatographia*. 2007, 66(11), 9009–9013. ISSN 0009-5893.

61. GUSTAVSSON, J., Cederberg, C., & Sonesson, U. Global food losses and food waste. Extent, Causes and Prevention. *Food and Agriculture Organisation*. p. 30, ISBN 978-92-5-107205-9.

62. HALLIWELL, B. Antioxidant characterization. Methodology and mechanism. *Biochemical Pharmacology*. 2011, 49(10), 1341–1348. ISSN 0006-2952.

63. HALLIWELL, B., & Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*. Oxford, UK: Oxford University press. 2005.

64. HANRAHAN, G., & Lu, K. Application of Factorial and Response Surface Methodology in Modern Experimental Design and Optimization. *Critical Reviews in Analytical Chemistry*. 2006, 36(3-4), 141–151. ISSN 1040-8347.

65. HEMERY, Y. M., Anson, N. M., Havenaar, R., Haenen, G. R. M. M., Noort, M. W. J., & Rouau, X. Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Research International*. 2010, 43(5), 1429–1438. ISSN 0963-9969.

66. HERRERO, M., Castro-Puyana, M., Mendiola, J. A., & Ibanez, E. Compressed fluids for the extraction of bioactive compounds. *TrAC - Trends in Analytical Chemistry*. 2013, 43(2), 67–83. ISSN 0165-9936.

67. HERRERO, M., Cifuentes, A., & Ibanez, E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review. *Food Chemistry*. 2006, 98(1), 136–148. ISSN 0308-8146.

68. HOSENEY, R. C., & Seib, P. A. Structural differences in hard and soft wheat. *Baker's Dig*. 1974, 47(6), 26–28.

69. HOSSAIN, M. B., Barry-Ryan, C., Martin-Diana, A. B., & Brunton, N. P. Optimisation of accelerated solvent extraction of antioxidant compounds from rosemary (Rosmarinus officinalis L.), marjoram (Origanum majorana L.) and oregano (Origanum vulgare L.) using response surface methodology. *Food Chemistry*. 2011, 126(1), 339–346. ISSN 0308-8146.

70. HRBAC, J., & Kohen, R. Biological redox activity: Its importance, methods for its quantification and implication for health and disease. *Drug Development Research*. 2000 50(3-4), 516–527. ISSN 1098-2299.

71. HUANG, D., Boxin, O. U., & Prior, R. L. The chemistry behind antioxidant capacity

assays. *Journal of Agricultural and Food Chemistry*. 2005, 53(6), 1841-1856. ISSN 0021-8561.

72. HUANG, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry*. 2002, 50(7), 1815–1821. ISSN 0021-8561.

73. IKUTA, K., Hashimoto, K., Kaneko, H., Mori, S., Ohashi, K., & Suzutani, T. Anti-viral and anti-bacterial activities of an extract of blackcurrants (Ribes nigrum L.). *Microbiology and Immunology*. 2012, 56(12), 805–809. ISSN 1684-1182.

74. JOMOVA, K., & Valko, M. Advances in metal-induced oxidative stress and human disease. *Toxicology*. 2011, 283(2-3), 65-87. ISSN 0300-483X.

75. JUNG, G., Uddin, S., Kwon, K., & Chun, B. Comparison of supercritical and near-critical carbon dioxide extraction of carotenoid enriched wheat bran oil. *African Journal of Biotechnology*. 2010, 9(45), 7702–7709. ISSN 1684-5315.

76. JUNG, G. W., Kang, H. M., & Chun, B. S. Characterization of wheat bran oil obtained by supercritical carbon dioxide and hexane extraction. *Journal of Industrial and Engineering Chemistry*. 2012, 18(1), 360–363. ISSN 1226-086X.

77. JURGEN, F., Maurizio, P., & Lester, P. *Redox-Genome Interactions in Health and Disease*. (CRC Press, Ed.). p. 640, 2003. ISBN 0824706889

78. JURGONSKI, A., Juśkiewicz, J., Zduńczyk, Z., Matusevicius, P., & Kołodziejczyk, K. Polyphenol-rich extract from blackcurrant pomace attenuates the intestinal tract and serum lipid changes induced by a high-fat diet in rabbits. *European Journal of Nutrition*. 2014, 53(8), 1603–1613. ISSN 1436-6207.

79. KAMAL-ELDIN, A., Lærke, H. N., Knudsen, K. E. B., Lampi, A. M., Piironen, V., Adlercreutz, H., Åman, P. Physical, microscopic and chemical characterisation of industrial rye and wheat brans from the Nordic countries. *Food and Nutrition Research*. 2009, 53, 1–11. ISSN 2333-1119.

80. KAPASAKALIDIS, P. G., Rastall, R. A., & Gordon, M. H. Extraction of polyphenols from processed black currant (Ribes nigrum L.) residues. *Journal of Agricultural and Food Chemistry*. 2006, 54(11), 4016–4021. ISSN 0021-8561.

81. KAPASAKALIDIS, P. G., Rastall, R. A., & Gordon, M. H. Effect of a cellulase treatment on extraction of antioxidant phenols from black currant (Ribes nigrum L.) pomace. *Journal of Agricultural and Food Chemistry*. 2009, 57(10), 4342–4351. ISSN 0021-8561.

82. KATINA, K., Laitila, A., Juvonen, R., Liukkonen, K. H., Kariluoto, S., Piironen, V., Poutanen, K. Bran fermentation as a means to enhance technological properties and bioactivity of rye. *Food Microbiology*. 2007, 24(2), 175–186. ISSN 1095-9998.

83. KEMZURAITE, A., Venskutonis, P. R., Baranauskiene, R., & Navikiene, D. Optimization of supercritical CO2 extraction of different anatomical parts of lovage (Levisticum officinale Koch.) using response surface methodology and evaluation of extracts composition. *Journal of Supercritical Fluids*. 2014, 87, 93–103. ISSN 0896-8446.

84. KO, S.-N., Kim, C.-J., Kim, H., Kim, C.-T., Chung, S.-H., Tae, B.-S., & Kim, I.-H. Tocol levels in milling fractions of some cereal grains and soybean. *Journal of the American*

Oil Chemists' Society. 2003, 80(6), 585–589. ISSN 1558-9331.

85. KOHEN, R., & Nyska, A. Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification. *Toxicologic Pathology*. 2002, 30(6), 620–650. ISSN 1533-1601.

86. KOSMALA, M., Kołodziejczyk, K., Markowski, J., Mieszczakowska, M., Ginies, C., & Renard, C. M. G. C. Co-products of black-currant and apple juice production: Hydration properties and polysaccharide composition. *LWT - Food Science and Technology*. 2010, 43(1), 173–180. ISSN 0023-6438.

87. KOSMALA, M., Zduńczyk, Z., Karlińska, E., & Juśkiewicz, J. The effects of strawberry, black currant, and chokeberry extracts in a grain dietary fiber matrix on intestinal fermentation in rats. *Food Research International*. 2014, 64, 752–761. ISSN 0963-9969.

88. KRAUJALIS, P., & Venskutonis, P. R. Optimisation of supercritical carbon dioxide extraction of amaranth seeds by response surface methodology and characterization of extracts isolated from different plant cultivars. *Journal of Supercritical Fluids*. 2013a, 73, 80–86. ISSN 0896-8446.

89. KRAUJALIS, P., & Venskutonis, P. R. Supercritical carbon dioxide extraction of squalene and tocopherols from amaranth and assessment of extracts antioxidant activity. *Journal of Supercritical Fluids*. 2013b, 80, 78–85. ISSN 0896-8446.

90. KRISHNA, A. G. G., Hemakumar, K. H., & Khatoon, S. Study on the Composition of Rice Bran Oil and Its Higher Free Fatty Acids Value. *Journal of the American Oil Chemists' Society*. 2006, 83(2), 117–120. ISSN 1558-9331.

91. KRYZEVICIUTE, N., Kraujalis, P., & Venskutonis, P. R. Optimization of high pressure extraction processes for the separation of raspberry pomace into lipophilic and hydrophilic fractions. *Journal of Supercritical Fluids*. 2016, 108, 61–68. ISSN 0896-8446.

92. KUMAR, G. S., & Krishna, A. G. G. Studies on the nutraceuticals composition of wheat derived oils wheat bran oil and wheat germ oil. *Journal of Food Science and Technology*. 2013, 52(2), 1145–1151. ISSN 0022-1155.

93. KWON, K. T., Uddin, M. S., Jung, G. W., Sim, B. S., & Chun, B. S. Supercritical carbon dioxide extraction of phenolics and tocopherols enriched oil from wheat bran. *World Academy of Science, Engineering and Technology.*2010, 64, 255–260. ISSN 2010-3778.

94. LANDBERG, R., Dey, E. S., Francisco, J. D. C., Aman, P., & Kamal-Eldin, A. Comparison of supercritical carbon dioxide and ethyl acetate extraction of alkylresorcinols from wheat and rye. *Journal of Food Composition and Analysis*. 2007, 20(6), 534–538. ISSN 0889-1575.

95. LANDBERG, R., Marklund, M., Kamal-Eldin, A., & Aman, P. An update on alkylresorcinols - Occurrence, bioavailability, bioactivity and utility as biomarkers. *Journal of Functional Foods.*2014, 7(1), 77–89. ISSN 1756-4646.

96. LANDBO, A. K., & Meyer, A. S. Enzyme-assisted extraction of antioxidative phenols from black currant juice press residues (Ribes nigrum). *Journal of Agricultural and Food Chemistry*. 2001, 49(7), 3169–3177. ISSN 0021-8561.

97. LEMPEREUR, I., Rouau, X., & Abecassis, J. Genetic and Agronomic Variation in Arabinoxylan and Ferulic Acid Contents of Durum Wheat (Triticum durumL.) Grain and Its

Milling Fractions. *Journal of Cereal Science*. 1997, 25(2), 103–110. ISSN 0733-5210.

98. LI, B. B., Smith, B., & Hossain, M. M. Extraction of phenolics from citrus peels: I. Solvent extraction method. *Separation and Purification Technology*. 2006, 48(2), 182–188. ISSN 1383-5866.

99. LI, J., Guo, Y., & Li, Z. Supercritical Carbon Dioxide and Hexane Extraction of Wax from Apple Peel Pomace: Content, Composition, and Thermal Properties. *Separation Science and Technology*. 2015, 50(14), 2230–2237. ISSN 0149-6395

100. MATTILA, P., Pihlava, J. M., & Hellström, J. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *Journal of Agricultural and Food Chemistry*. 2005, 53(21), 8290–8295. ISSN 0021-8561.

101. MENDIOLA, J. A., Herrero, M., Cifuentes, A., & Ibanez, E. Use of compressed fluids for sample preparation: Food applications. *Journal of Chromatography A.* 2007, 1152(1-2), 234–246. ISSN 0021-9673.

102. MEYER, A. S. Enzyme technology for precision functional food ingredient processes. *Annals of the New York Academy of Sciences.*2010, 1190, 126–132. ISSN 0077-8923

103. MILLER, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*. 1993, 84(4), 407–412. ISSN 0143-5221.

104. MOYER, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. *Journal of Agricultural and Food Chemistry*. 2002, 50(3), 519–525. ISSN 0021-8561.

105. NAWIRSKA, A., & Kwaśniewska, M. Dietary fibre fractions from fruit and vegetable processing waste. *Food Chemistry*. 2005, 91(2), 221–225. ISSN 0308-8146.

106. NORDLUND, E., Heiniö, R. L., Viljanen, K., Pihlava, J. M., Lehtinen, P., & Poutanen, K. Flavour and stability of rye grain fractions in relation to their chemical composition. *Food Research International*. 2013, 54(1), 48–56. ISSN 0963-9969.

107. NOUR, V., Trandafir, I., & Cosmulescu, S. Antioxidant capacity, phenolic compounds and minerals content of blackcurrant (ribes nigrum L.) leaves as influenced by harvesting date and extraction method. *Industrial Crops and Products*. 2014, 53, 133–139. ISSN 0926-6690.

108. OU, B., Hampsch-Woodill, M., & Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry*. 2001, 49(10), 4619–4626. ISSN 0021-8561.

109. OU, S., & Kwok, K. C. Ferulic acid: Pharmaceutical functions, preparation and applications in foods. *Journal of the Science of Food and Agriculture*. 2004, 84(11), 1261-1269. ISSN 1097-0010.

110. OUFNAC, D. S., Xu, Z., Sun, T., Sabliov, C., Prinyawiwatkul, W., & Godber, J. S. Extraction of antioxidants from wheat bran using conventional solvent and microwave-assisted methods. *Cereal Chemistry*. 2007, 84(2), 125–129. ISSN 0009-0352.

111. PANDEY, K. B., & Rizvi, S. I. Plant polyphenols as dietary antioxidants in human

health and disease. *Oxidative Medicine and Cellular Longevity*. 2009, 2(5), 270–278. ISSN 1942-0900.

112. PATEL, S. Cereal bran: the next super food with significant antioxidant and anticancer potential. *Mediterranean Journal of Nutrition and Metabolism*. 2012, 5(2), 91–104. ISSN 1973-7998.

113. PIESZKA, M., Migdał, W., Gąsior, R., Rudzińska, M., Bederska-Łojewska, D., Pieszka, M., & Szczurek, P. Native oils from apple, blackcurrant, raspberry, and strawberry seeds as a source of polyenoic fatty acids, tocochromanols, and phytosterols: A health implication. *Journal of Chemistry*. 2015, 1-8. ISSN 2090-9063.

114. PINELO, M., Arnous, A., & Meyer, A. S. Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release. *Trends in Food Science and Technology*. 2006, 17(11), 579-590. ISSN 0924-2244.

115. POURALI, O., Asghari, F. S., & Yoshida, H. Production of phenolic compounds from rice bran biomass under subcritical water conditions. *Chemical Engineering Journal*. 2010, 160(1), 259–266. ISSN 1385-8947.

116. PRIOR, R. L., & Cao, G. In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radic Biol Med*. 1999, 27(11-12), 1173–1181. ISSN 0891-5849.

117. PRIOR, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry*. 2003, 51(11), 3273–3279. ISSN 0021-8561.

118. PRIOR, R., Wu, X., & Schaich, K. Standarized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *Journal of Agricultural and Food Chemistry*. 2005, *53*(10), 4290–4302. ISSN 0021-8561.

119. PRONYK, C., & Mazza, G. Design and scale-up of pressurized fluid extractors for food and bioproducts. *Journal of Food Engineering*. 2009, 95(2), 215-226. ISSN 0260-8774.

120. PRUCKLER, M., Siebenhandl-Ehn, S., Apprich, S., Höltinger, S., Haas, C., Schmid, E., & Kneifel, W. Wheat bran-based biorefinery 1: Composition of wheat bran and strategies of functionalization. *LWT - Food Science and Technology*. 2014, 56(2), 211–221. ISSN 0023-6438.

121. RUI, da Silva, R. P. F. F., Rocha-Santos, T. A. P., & Duarte, A. C. Supercritical fluid extraction of bioactive compounds. *TrAC Trends in Analytical Chemistry*. 2016, 76, 40–51. ISSN 0165-9936.

122. PULIDO, R., Bravo, L., & Saura-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry*. 2000, 48(8), 3396–3402. ISSN 0021-8561.

123. PURI, M., Sharma, D., & Barrow, C. J. Enzyme-assisted extraction of bioactives from plants. *Trends in Biotechnology*. 2012, 30(1), 37-44. ISSN 0167-7799.

124. RAVINDRAN, R., & Jaiswal, A. K. Exploitation of Food Industry Waste for High-Value Products. *Trends in Biotechnology*. 2015, 34(1), 58–69. ISSN 0167-7799.

125. RE, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C.

Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*. 1999, 26(9-10), 1231–1237. ISSN 0891-5849.

126. REBOLLEDA, S., Beltran, S., Sanz, M. T., Gonzalez-Sanjose, M. L., & Solaesa, angela G. Extraction of alkylresorcinols from wheat bran with supercritical CO2. *Journal of Food Engineering*. 2013, 119(4), 814–821. ISSN 0260-8774.

127. REBOLLEDA, S., Beltran, S., Teresa Sanz, M., & Luisa Gonzalez-Sanjose, M. Supercritical fluid extraction of wheat bran oil: Study of extraction yield and oil quality. *European Journal of Lipid Science and Technology*. 2014, 116(3), 319–327. ISSN 1438-7697.

128. ROGINSKY, V., & Lissi, E. A. Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry*. 2005, 92(2), 235–254. ISSN 0308-8146.

129. ROJ, E., Dobrzynska-Inger, A. Kostrzewa, D., Kolodziejczyk, K., Sojka, M., Krol, B., Miszczak, A., & Markowski, J. Extraction of berry seed oils with supercritical CO2. *Przemysl Chemiczny*. 2009, 88(12), 1325–1330. ISSN 0033-2496.

130. ROJ, E., & Dobrzyńska-Inger, A. Industrial use of supercritical extraction in processing of plant materials — Status and prospects. *Pamiętnik Puławski*. 2009, 151, 608–616. ISSN 0552-9778.

131. ROMANIK, G., Gilgenast, E., Przyjazny, A., & Kaminski, M. Techniques of preparing plant material for chromatographic separation and analysis. *Journal of Biochemical and Biophysical Methods*. 2007, 70(2), 253-261. ISSN 0165-022X.

132. ROSA, N. N., Barron, C., Gaiani, C., Dufour, C., & Micard, V. Ultra-fine grinding increases the antioxidant capacity of wheat bran. *Journal of Cereal Science*. 2013, 57(1), 84–90. ISSN 0733-5210.

133. ROSENBLAT, M., Volkova, N., Attias, J., Mahamid, R., & Aviram, M. Composition of polyphenolic-rich beverages (mostly pomegranate and black currant juices) by health subjects for a short term increased serum antioxidant status, and the serum's ability to attenuate macrophage cholesterol accumulation. *Food & Function*. 2010, 1, 99–109. ISSN 2042-6496.

134. ROSTAGNO, M. A., Arrigo, M., Martinez, J. A., & Martinez, J. A. Combinatory and hyphenated sample preparation for the determination of bioactive compounds in foods. *TrAC - Trends in Analytical Chemistry*. 2010, 29(6), 553-561. ISSN 0165-9936.

135. RUIZ DEL CASTILLO, M. L., Dobson, G., Brennan, R., & Gordon, S. Fatty Acid Content and Juice Characteristics in Black Currant (Ribes nigrum L.) Genotypes. *Journal of Agricultural and Food Chemistry*. 2004, 52(4), 948–952. ISSN 0021-8561.

136. SAHOO, P., Barman, T., & Davim, J. P. *Fractal Analysis in Maschining.* Springer Briefs in Applied Sciences and Technology. 2011, p.121. ISBN 3642179215.

137. SANDELL, M., Laaksonen, O., Ja, R., Rostiala, N., Pohjanheimo, T., Tiitinen, K., & Kallio, H. Orosensory profiles and chemical composition of black currant (ribes nigrum) juice and fractions of press residue. *Journal of Agricultural and Food Chemistry*. 2009, 57(9), 3718–3728. ISSN 0021-8561.

138. SAULNIER, L., Sado, P. E., Branlard, G., Charmet, G., & Guillon, F. Wheat arabinoxylans: Exploiting variation in amount and composition to develop enhanced

varieties. *Journal of Cereal Science*. 2007, 46(3), 261–281. ISSN 0733-5210.

139. SEABRA, I. J., Braga, M. E. M., Batista, M. T. P., & de Sousa, H. C. Fractioned high pressure extraction of anthocyanins from elderberry (Sambucus nigra L.) pomace. *Food and Bioprocess Technology*. 2010, 3(5), 674–683. ISSN 1935-5130.

140. SHAHIDI, F., & Zhong, Y. Measurement of antioxidant activity. *Journal of Functional Foods*. 2015, 18, 757–781. ISSN 1756-4646.

141. SHAPIRO, M. Redox balance in the body: An approach to quantification. *Journal of Surgical Research*. 1972, 3, 138–152. ISSN 0022-4804.

142. SHEWRY, P. R. Principles of Cereal Science and Technology. *Journal of Cereal Science*. 2010, 51(3), 415. ISSN 0733-5210.

143. SINGLETON, V. L., Orthofer, R., & Lamuela-Raventes, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*. 1998, 299, 152–178. ISSN 0076-6879.

144. SINGLETON, V. L., Rossi Jr., J. A., & Rossi J A Jr. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*. 1965, 16(3), 144–158. ISSN 0002-9254.

145. SOARES, B. M. C., Gamarra, F. M. C., Paviani, L. C., Gonçalves, L. A. G., & Cabral, F. A. Solubility of triacylglycerols in supercritical carbon dioxide. *Journal of Supercritical Fluids*. 2007, 43(1), 25–31. ISSN 0896-8446.

146. SOJKA, M., Guyot, S., Kolodziejczyk, K., Krol, B., & Baron, A. Composition and properties of purified phenolics preparations obtained from an extract of industrial blackcurrant (Ribes nigrum L.) pomace. *Journal of Horticultural Science and Biotechnology*. 2009, 84(6), 100–106. ISSN 1462-0316.

147. SOJKA, M., & Król, B. Composition of industrial seedless black currant pomace. *European Food Research and Technology*. 2009, 228(4), 597–605. ISSN 1438-2377

148. SOVOVA, H. Steps of supercritical fluid extraction of natural products and their characteristic times. *The Journal of Supercritical Fluids*. 2012, 66, 73–79. ISSN 0896-8446.

149. SOVOVA, H., Zarevúcka, M., Vacek, M., & Stránský, K. Solubility of two vegetable oils in supercritical CO2. *Journal of Supercritical Fluids*. 2001, 20(1), 15–28. ISSN 0896-8446.

150. SOWBHAGYA, H. B., & Chitra, V. N. Enzyme-assisted extraction of flavorings and colorants from plant materials. *Critical Reviews in Food Science and Nutrition*. 2010, 50(2), 146–161. ISSN 1040-8398.

151. TALCOTT, S. T. *Berry fruit: Value-added products for health promotion*. CRC Press. 2007, p. 442. ISBN 9780849358029.

152. TOMITA, K., Machmudah, S., Wahyudiono, Fukuzato, R., Kanda, H., Quitain, A. T., Goto, M. Extraction of rice bran oil by supercritical carbon dioxide and solubility consideration. *Separation and Purification Technology*. 2014, 125, 319–325. ISSN 1383-5866.

153. TSAO, R., & Deng, Z. Separation procedures for naturally occurring antioxidant phytochemicals. *Journal of Chromatography B: Analytical Technologies in the Biomedical*

and Life Sciences. 2004, 812(1-2), 85–99. ISSN 1570-0232.

154. VITAGLIONE, P., Napolitano, A., & Fogliano, V. Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. *Trends in Food Science and Technology*. 2008, 19(9), 451-463. ISSN 0924-2244.

155. VUORINEN, A. L., Kalpio, M., Linderborg, K. M., Hoppula, K. B., Karhu, S. T., Yang, B., & Kallio, H. P. Triacylglycerol biosynthesis in developing Ribes nigrum and Ribes rubrum seeds from gene expression to oil composition. *Food Chemistry*. 2016, 196, 976–987. ISSN 2308-7943.

156. WANG, J., Sun, B., Cao, Y., Tian, Y., & Li, X. Optimisation of ultrasound-assisted extraction of phenolic compounds from wheat bran. *Food Chemistry*. 2008, 106(2), 804–810. ISSN 2308-7943.

157. WIERINGA G. V. On the Occurrence of Growth Inhibiting Substances in Rye. Wageningen, H. Veenman. 1967, p. 68. ASIN B0006BYH4S.

158. WIJNGAARD, H., Hossain, M. B., Rai, D. K., & Brunton, N. Techniques to extract bioactive compounds from food by-products of plant origin. *Food Research International*. 2012, 46(2), 505–513. ISSN 0963-9969.

159. XIE, X. (Sherry), Cui, S. W., Li, W., & Tsao, R. Isolation and characterization of wheat bran starch. *Food Research International*. 2008, 41(9), 882–887. ISSN 0963-9969.

160. YANG, B., Ahotupa, M., Määttä, P., & Kallio, H. Composition and antioxidative activities of supercritical CO2-extracted oils from seeds and soft parts of northern berries. *Food Research International*. 2011, 44(7), 2009–2017. ISSN 0963-9969.

161. YILMAZ, I. Effects of rye bran addition on fatty acid composition and quality characteristics of low-fat meatballs. *Meat Science*. 2004, 67(2), 245–249. ISSN 0309-1740.

162. ZARNOWSKI, R., & Suzuki, Y. Expedient Soxhlet extraction of resorcinolic lipids from wheat grains. *Journal of Food Composition and Analysis*. 2004, 17(5), 649–663. ISSN 0889-1575.

163. ZHOU, K., & Yu, L. Effects of extraction solvent on wheat bran antioxidant activity estimation. *LWT - Food Science and Technology*. 2004, 37(7), 717–721. ISSN 0023-6438.

164. ZUKNIK, M. H., Nik Norulaini, N. A., & Mohd Omar, A. K. Supercritical carbon dioxide extraction of lycopene: A review. *Journal of Food Engineering*. 2012, 112(4), 253-262. ISSN 0260-8774.

LIST OF PUBLICATIONS ON THE TOPIC OF DISSERTATION

Publications corresponding to the list of the Institute of Science Information (ISI) database

1. **Povilaitis, D.**, Venskutonis, P.R. Optimization of supercritical carbon dioxide extraction of rye bran using response surface methodology and evaluation of extract properties // The Journal of Supercritical Fluids, ISSN 0896-8446, 2015, vol. 100, p. 194–200. IF: 2.371 (2014).

2. **Povilaitis, D.**, Šulniūtė, V., Venskutonis, P.R., Kraujalienė, V. Antioxidant properties of wheat and rye bran extracts obtained by pressurized liquid extraction with different solvents. Journal of Cereal Science, ISSN 0733-5210, 2015, vol. 62, p. 117-123. IF: 2.094 (2014).

List of the presentations in the international conferences

1. Kitrytė, Vaida**; Povilaitis, Darius**; Kraujalienė, Vaida; Šulniūtė, Vaida; Pukalskas Audrius; Venskutonis, Petras Rimantas. Biorefining of sea buckthorn pomace and seeds into valuable components by using high pressure and enzyme-assisted extraction methods // Fruit and vegetable processing: 2nd Euro-Mediterrranean symposium on fruit and vegetable processing, April 4-6, 2016. Avignon, France: book of abstracts / INRA – National Institute for Agricultural Research, University of Avignon, France. p. 148.
2. **Povilaitis, Darius**; Kitrytė, Vaida; Venskutonis, Petras Rimantas, Dursun, Derya; Schulte, Johanna. Biorefining of rye bran by high pressure extraction technologies // FOODBALT – 2015: 10th Baltic conference on food science and technology „Future food: innovations, science and technology“, May 21−22, 2015, Kaunas, Lithuania: abstract book / Kaunas University of Technology, Kaunas: KTU, 2015. p. 25. ISBN 9786090211380.
3. Basegemez, Hatice Imge Oktay; Alasalvar, Cesarettin; **Povilaitis, Darius**; Venskutonis, Petras Rimantas. Optimization of supercritical SC-CO_2 extraction of *Ribes nigrum* L. berry pomace using response surface methodology // FOODBALT – 2015: 10th Baltic conference on food science and technology „Future food: innovations, science and technology“, May 21−22, 2015, Kaunas, Lithuania: book of abstracts / Kaunas University of Technology, Kaunas: KTU, 2015. p. 16. ISBN 9786090211380.
4. **Povilaitis, Darius**; Venskutonis, Petras Rimantas. Optimization of rye bran supercritical CO_2 extraction by response surface methodology // ISEKI Food 2014: 3rd international conference “Food science and technology excellence for sustainable bioeconomy, May 21−23, 2014. Athens, Greece: book of abstracts / ISEKI Food Association, Athens: National Technical University of Athens, 2015. p. 87. ISBN 9789608978980.
5. **Povilaitis, Darius**; Venskutonis, Petras Rimantas. Extraction of bioactive compounds from rye bran using different extraction techniques // FOODBALT – 2014: 9th Baltic conference on food science and technology „Food for consumer

well-being“, May 8−9, 2014, Jelgava, Latvia: book of abstracts / Latvia University of Agriculture, Jelgava, 2014. p. 133. ISSN 2255-9809.

6. Venskutonis, Petras Rimantas; Šulniūtė, Vaida; **Povilaitis, Darius**; Jaime, Isabel; Rovira, Jordi. Rye and wheat bran extracts – a source of functional ingredients // Functional foods, nutraceuticals, natural health products, and dietary supplements: 2014 Annual conference and exhibition. October 14−17, 2014, Istanbul, Turkey: book of abstracts /. International Society for Nutraceuticals and Functional Foods (ISNFF). p. 357.

Other presentations

1. Povilaitis, Darius; Venskutonis, Petras Rimantas. Uogų išspaudų biorafinavimas į vertingas funkcines medžiagas. // „Technorama‘2016“ jaunųjų mokslininkų paroda-konkursas, Kauno technologijos universitetas, Kaunas. Darbų katalogas, p. 149−150.

SL344. 2016-11-14, 13 leidyb. apsk. l. Tiražas 12 egz. Užsakymas 415 .

Išleido Kauno technologijos universitetas, K. Donelaičio g. 73, 44249 Kaunas
Spausdino leidyklos „Technologija“ spaustuvė, Studentų g. 54, 51424 Kaunas