KAUNAS UNVERSITY OF TECHNOLOGY

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OPTIMISATION OF FRACTIONATION PROCESS OF AMARANTH (*AMARANTHUS* SPP.) BY APPLYING HIGH PRESSURE EXTRACTION METHODS

Doctoral Dissertation
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Scientific Supervisor:
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KAUNO TECHNOLOGIJOS UNIVERSITETAS

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BURNOČIO (*AMARANTHUS* SPP.) FRAKCIONAVIMO PROCESŲ OPTIMIZAVIMAS TAIKANT DIDELIO SLĖGIO EKSTRAKCIJOS BŪDUS

Daktaro disertacija
Technologijos mokslai, chemijos inžinerija (05T)

2014, Kaunas

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TABLE OF CONTENT

1. LIST OF ABBREVIATIONS

$ABTS^{\bullet+}$	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation
ANOVA	Analysis of variance
AS	Analytical scale
AscA	Ascorbic acid
ASE	Accelerated solvent extraction
CCD	Central composite design
CERP	Constant extraction rate period
DCRP	Diffusion controlled rate period
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
FA	Fatty acid
FAME	Fatty acid methyl ester
FERP	Falling extraction rate period
FID	Flame ionisation detector
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
GC	Gas chromatography
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
IDF	Insoluble dietary fibre
LC	Liquid chromatography
M_{CERP}	Mass-transfer rate during CERP
MS	Mass spectrometry
MSD	Mass selective detector
OEC	Overall extraction curve
OEC	Overall extraction curve
PAHs	Polycyclic aromatic hydrocarbons
PHWE	Pressurised hot water extraction
PLE	Pressurised liquid extraction
PS	Pilot scale
PUFA	Poly unsaturated fatty acids
QTOF	Quadrupole time-of-flight
R_{CERP}	Yield of extract during CERP
RSC	Radical scavenging capacity
RSD	Relative standard deviation
RSM	Response surface methodology
SAF	Supercritical antisolvent fractionation
$SC\text{-}CO_2$	Supercritical carbon dioxide
SC-CO2	Supercritical carbon dioxide
$SCE\text{-}CO_2$	Supercritical extraction carbon dioxide
SDF	Soluble dietary fibre
SFE	Supercritical fluid extraction
SL	Standard litre at standard state (P_{CO2}=100 kPa, T_{CO2}=20 °C, ρ_{CO2}=0.0018 g/ml)

SWE	Subcritical water extraction
t_{CERP}	Time of CERP
TDF	Total dietary fibre
TEAC	Trolox equivalent antioxidant capacity
UPLC	Ultra high performance liquid chromatography

I. INTRODUCTION

Relevance of the research. The search for bioactive compounds of natural origin has arisen in the past two decades. Consideration of raw material as well as environmentally-friendly process application is of importance. In last decades, supercritical fluid technology has gained much interest in pharmaceutical and food sectors because of an increased preference for natural products and regulations related to toxicity levels of the natural bioactive substances. Natural new generation products without residues of the chemicals have made supercritical fluid technology as an alternative to traditional solvent extraction for the extraction and fractionation of active ingredients. Different sources of bioactive compounds have been studied; plants, agricultural by-products and marine products being among the most promising, some have already been associated with lower risks of coronary heart diseases and cancer (Hooper, et al., 2008).

The supercritical carbon dioxide ($SC\text{-}CO_2$) as a solvent has been of choice for food applications. Advantage of processing with $SC\text{-}CO_2$ include: low processing temperatures, minimal thermal degradation of the bioactive components, ease of separation of extraction solvent, no solvent residue left in the product, processing in the CO_2 environment minimizes undesirable oxidation reactions, which is especially beneficial for the sensitive bioactive components of specialty oils such as sterols, tocols, carotenoids, and polyunsaturated fatty acids (PUFAs) (Temelli, et al., 2007). $SCE\text{-}CO_2$ was successfully applied as a separation and fractionation technique for reducing free fatty acid content, minimizing phytosterols loss (Dunford & King, 2000) and for obtaining the fractions enriched with tocopherols and tocotrienols (Sarmento, et al., 2006).

The genus *Amaranthus* includes approximately 60 species growing in many areas of the world. Amaranth has been consumed for centuries as a green leafy vegetable, grain or used for ornamental purposes. *Amaranthus* is a gluten-free pseudocereal, also containing various valuable constituents and from these points of view it is attractive as a raw material for foods with health and medicinal benefits, including the prevention and treatment of some diseases and disorders. The importance of amaranth as a dietary supplement is associated to its nutritional value of lipid fraction which is related to its fatty acid profile, similar to that of corn oil and tocopherols which are known to protect fatty acids from oxidation (Bruni, et al., 2002). Tocopherols and tocotrienols are well-known natural antioxidant in oilseeds which can protect unsaturated lipids and cholesterol *in vivo* playing an important role in the body (Heinemann, et al., 1993). Vitamin E contents in amaranth seed oil were comparable to those in other grain crops (Brigelius-Flohé, et al., 2002). Eight forms of this vitamin can be found under natural conditions, namely: α-, β-, γ-, and δ-T, as well as α-, β-, γ-, and δ-T3 (tocotrienols).

Squalene as a minor component is present almost in all plant oils, whereas the oil of amaranth is one of the richest sources of squalene (2-8%). Triterpene squalene probably is the most important constituent of the unsaponifiable fraction of amaranth lipids possessing various beneficial effects on health as a cancer

chemopreventative substance, anticancerogenic and reducing serum cholesterol levels. Squalene is used in cosmetics, and more recently as an immunologic adjuvant in vaccines (Sun, et al., 1997).

Amaranth seeds also contain high quality proteins with amino acid composition close to the ideal protein (Yanez, et al., 1994).

The main aim of this research was to create exhaustive and effective extraction and fractionation process of anatomical parts and bioactive compounds of amaranth grown in Lithuania, applying high pressure extraction methods and rational raw material processing in order to obtain high value and different composition functional components. The following objectives were raised for achieving this aim:

1. To evaluate seed pretreatment for effective and exhaustive extraction.
2. To determine optimal parameters on the extraction efficiency of lipid fraction from amaranth seeds by using pressurised liquid extraction and to compare lipid yields and composition of amaranth seeds obtained from various sources in Lithuania.
3. To determine the optimal conditions (temperature, pressure, extraction time, CO_2 flow rate) for the extraction of amaranth oil using SCE-CO_2 and to test the established optimal parameters for the determination of oil yield, fatty acid composition and squalene content in various amaranth accessions.
4. To investigate the possibilities of extraction and fractionation of tocopherols and squalene from amaranth seeds by SCE-CO_2 using cosolvent ethanol and two separators and to evaluate the antioxidant activity of lipophilic extracts.
5. To evaluate antioxidant properties and characterize phytochemical composition of different anatomical parts (seeds, leaves, flowers, and stems) of locally grown *Amaranthus* spp.
6. To determine the optimal conditions for the extraction of rutin as the main flavonoid from amaranth leaves using PLE and to improve rutin separation using supercritical antisolvent fractionation in order to obtain fraction enriched with rutin.
7. To evaluate biorefinery possibilities of anatomical parts of amaranth in order to obtain functional high-added value components.

Scientific novelty and practical significance of the research. Every variety of plants is characterized by unique biological structure and the phytochemical composition of secondary metabolism products. The amount of bioactive compounds, even of the same variety, varies according to growing climatic conditions. For this reason it is very important to develop effective methods of isolation and fractionation of in Lithuania grown amaranth (*Amaranthus* spp.) components and bioactive compounds by applying traditional and modern methods as well as modelling their parameters, which should ensure rational and cost-effective processing of plant origin raw materials into high added value functional ingredients for various applications. Until now scarce comprehensive studies were performed for *Amaranthus* spp. grown in Lithuania. There were none performed

investigations using complex extraction and fractionation methods of amaranth. For achieving this aim the combinations of accelerated solvent extraction and supercritical fluid extraction were applied and such important variables of extraction and fractionation of plant raw materials as grinding (particles size), solvent polarity, temperature, pressure and duration of extraction processes, solvent flow rate, the number of extraction cycles, the concentration solvent modifying extraction system (in case of supercritical fluid extraction with fluid carbon dioxide) and the parameters of supercritical fluid extraction separators was determined.

Structure and outline of the dissertation. This dissertation is divided into five parts. Literature review related to theoretical background of supercritical fluids, modelling of SFE state as well as an overview of SFE applications are presented in Chapter 2. Chapter 2 also include information about raw material and its components analysed in this work. Description of materials, applied methods and experimental set-ups are summarized in Chapter 3. The results obtained by employing combinations of various extraction methods as well as experiments optimisation are described in Chapter 4. Chapter 5 summarizes the results of this work. In total 182 references were used. Dissertation also includes summary and list of 5 publications on the dissertation topic as well as 10 presentations at international conference. The final work contains 118 pages including 32 tables and 40 figures.

Publication of research results. The results of this research are presented in 5 publications, corresponding to the list of the Institute of Science Information (ISI) database, and reported at 8 international conferences.

Statements presented for the defence

1. Modelling of supercritical carbon dioxide extraction and pressurised liquid extraction of amaranth seeds applying central composite design and response surface methodology is effective method to determine optimal process parameters.
2. Complex application of pressurised liquid extraction and supercritical fluid extraction and fractionation processes is effective method obtaining high value squalene, tocopherols and rutin enriched amaranth fractions from various plant anatomical parts.

II. LITERATURE REVIEW

2.1. Supercritical fluid extraction

Supercritical State

A substance reaches supercritical state when pressure and temperature are beyond their respective critical values. When the substance reaches its critical value the properties of its gas and liquid phases converge and no distinction exists between the two phases becoming a homogeneous supercritical fluid. The critical point is defined as the end point of the vapor-pressure curve of the fluid, as presented in Figure 2.1 A. The critical point of the substance is called the point at which critical temperature and critical pressure meet (Figure 2.1 A).The properties of supercritical fluid above it‘s critical point with a small increase in pressure causes large increases in the density of the supercritical phase (Figure 2.1 B).

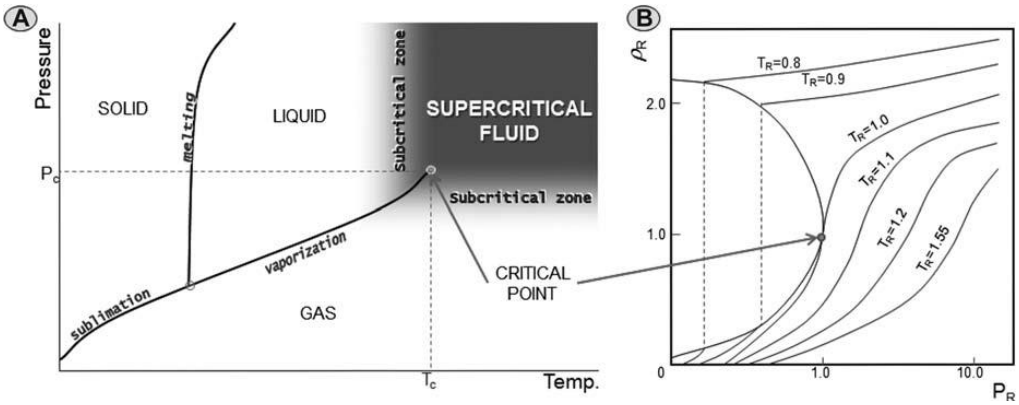


Figure 2.1. Carbon dioxide pressure - temperature phase diagram (A) and density - pressure phase diagram at different temperatures (B) considering reduced variables (Mendiola, et al., 2013)

The critical point has several peculiar properties:
There is no gas - liquid interface;
The isothermal compressibility becomes infinitely positive;
The coefficient of thermal expansion is infinite and positive;
The enthalpy of vaporization is zero;
If the density is constant and equal to the critical density, the heat capacity at constant volume tends to infinity;
The supercritical region also has properties that make it unique:
Density above the critical point depends on the pressure and the temperature, but in any case is closer to the liquid than the gas;
The viscosity is much lower than that of the liquid;
The very low surface tension enables a high permeation through porous solids and packed beds.
Currently are known five states of matter: solid, liquid, gas, plasma and Bose-Einstein condensate. The supercritical fluid is cuasi-state matter with intermediate

properties between liquid and gas. Due to these unique and advantageous features with gas-like transport properties, supercritical fluids are using in many applications (Table 2.1.).

Table 2.1. Physical properties of supercritical fluids (Brunner, 2005)

Fluid	*P/ T (MPa)/(K)*	*Density ρ (kg/m^3)*	*Diffusion coefficient D_{ij} (cm^2/s)*	*Viscosity η (g/cm·s)*
Gas	0.1/298	0.6-2.0	0.1-0.4	(1-3)x10^{-4}
SCF	Pc/Tc	200-500	0.7x10^{-3}	(1-3)x10^{-4}
Liquid	0.1/298	600-1600	2x10^{-6} -2x10^{-5}	(0.2-3)x10^{-2}

Supercritical solvents

There are many substances used as supercritical fluids, the critical points of most commonly used substance are presented in Table 2.2. Among the green solvents used in supercritical fluid extraction (SFE), carbon dioxide (CO_2) is the most commonly used supercritical fluid. In comparison to other substances, CO_2 presents a critical temperature (Tc) close to room temperature (31.1 °C) and a relative low critical pressure (Pc=7.38 MPa). Besides, it is relatively inexpensive, inert, environmentally friendly and recognized as safe (GRAS) also can be used in high purity, non-flammable, atoxic and non-explosive.

Table 2.2. Critical properties of substances most commonly used in supercritical conditions (Brunner, 2005)

Component	**Critical temperature Tc (°C)**	**Critical pressure Pc (MPa)**	**Critical density ρ (g/ml)**
Carbon dioxide	31.1	7.38	0.47
Ethane	32.3	4.87	0.2
Propane	96.8	4.25	0.22
n-Hexane	234.5	3.01	0.23
Acetone	235.1	4.70	0.28
Methanol	239.6	8.09	0.27
Ethanol	240.9	6.14	0.28
Water	374.1	22.06	0.32

SC-CO_2 is highly penetrating; its diffusivity could be easily changed by adjusting solvent strength. CO_2 is gaseous at room temperature and atmospheric pressure, thus providing solvent-free extracts. Another importance for food and natural products is the ability of SFE using CO_2 to be operated at low temperatures using a non-oxidant medium, which allows the extraction of thermally labile or easily oxidized compounds (Schutz, 2007). CO_2 is a non-polar solvent, what makes it unable to dissolve polar substances. The solubility of chemical substances in non-

polar solvents decreases with increasing molecular weight and mostly with increasing polarity and number of polar functional groups. Supercritical CO_2 tends to be selective towards lower molecular weight compounds (<250) or weakly polar groups such as lipids, cholesterol, aldehydes, ethers, esters and ketones, while high molecular weight (> 400) or polar groups such as hydroxyl, carboxyl, and sugars, polysaccharides, amino acids, proteins, phosphatides, glycosides, inorganic salts, are relatively insoluble in dense carbon dioxide (Table 2.3).

Table 2.3. Solubility of different compounds in supercritical CO_2 (Moyler, 1993)

Highly Soluble	Moderately Soluble	Almost Insoluble
Organic compounds of low polarity and low molecular weight (< 250)	Polar organic compounds of molecular weight lower than 400	Highly polar organic compounds of molecular weight above 400
Highly volatile substances, used for aromas and flavouring in foods	Substances with low volatility	Non-volatile substances
Thiols, pyrazines, thiazoles, acetic acid, benzaldehyde, hexanol, glycerol and acetates	Water, terpenes, oleic acid, glycerol and saturated lipids with chains of up to 12 carbons	Proteins, sugars, olysaccharides, amino acids, inorganic salts, nitrates, waxes

In order to increase the efficiency of the process, small percentages of polar modifiers (ethanol or others alcohols) can be employed. There are two main procedures to study co-solvents and modifiers: accounts for a mixing of the modifier with the CO_2 flow and mixes the modifier with raw material in the extraction cells. Last procedure is always associated to a static extraction step in which the modifier, in contact with the sample matrix is able to substitute the analyte molecules bound in active centers of the matrix and release them into the supercritical fluid phase (Rozzi & Singh, 2002).

Solubility in supercritical fluids

Operating pressure and temperature highly influence the yield of a solute and the separation selectivity. The solvent capacity increase with pressure at constant temperature, thus increase the yield of solute. In general terms, increasing the pressure leads to an exponential increase of the solubility due to increase of density of the solvent. The components with high vapour pressure have higher solubility in a supercritical medium. Other important aspects influencing solubility of components in the SCF are their polarity and molecular weight as well as extraction temperature. Solubility of the components is lowered as the polarity and/or the molecular weights of the solutes are increased.

For an isobaric system, an increase in the temperature promotes two opposite effects: it reduces the solvent power of CO_2 by a decrease of the density, thus decreasing the solute solubility and, on the other hand, it increases the vapour pressure of solute which can be more easily transferred to the supercritical phase over certain ranges of pressure. The solubility in the subcritical fluid at constant pressure increases up to temperatures slightly below the Tc, a further increase in temperature, at low pressures, leads to a decrease of the low-volatility substance

solubility and at high pressures still to an increase (Fig. 2.2). High and low pressures refer to a medium pressure is about 10MPa. This anomalous behaviour when the solubility of the solute decreases with a temperature increase is called the retrograde behaviour.

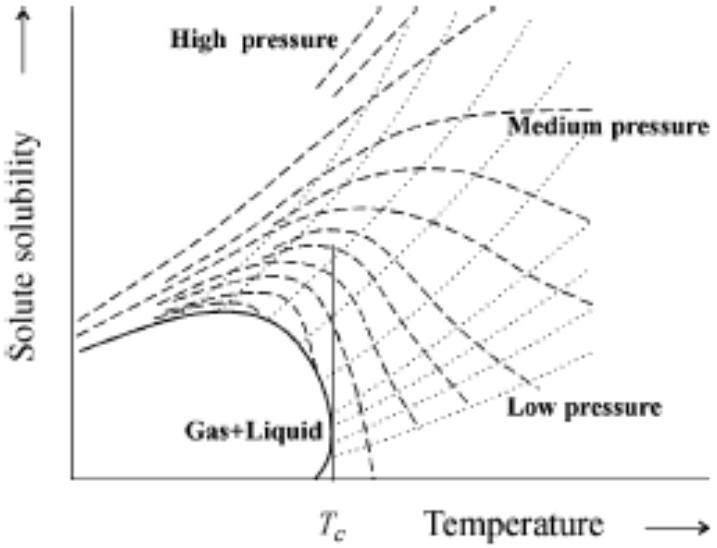


Figure 2.2. Variations in the solubility of a low-volatility substances (liquid or solid) in a subcritical (T < Tc) or supercritical fluid (T > Tc) as a function of process temperature, process pressure (segmented lines), and solvent density (doted lines) (Brunner, 1994)

Phase equilibrium engineering plays a key role in the development and design of supercritical extraction processes. Since equilibrium is achieved, it can provide valuable information about the dissolution capacity of a determined solvent, the amount of solvent dissolved in the liquid phase and the equilibrium composition of the liquid phase (Brunner, 1994). The equilibrium is achieved when temperature, pressure and chemical potential composed by n components and π coexisting phases are equalized: $T^{(1)}= T^{(2)}= \ldots = T^{(\pi)}$; $P^{(1)}= P^{(2)}= \ldots = P^{(\pi)}$; $\mu^{(1)}= \mu^{(2)}= \ldots = \mu^{(\pi)}$. Equilibrium predictions in systems having two or more fluid phases are more complex than those in cases of solid solubility's (Sandler, 1989).

In order to explain squalene solubility in SC-CO_2 Bhattacharjee, et al., (2012) has been used Chrastil equation which showed good agreement with the experimentally obtained yields. This equation showed a linear relationship between the logarithm of solubility of a solute and the logarithm of SC-CO_2 density.

When dealing with really complex matrices or extracting different components (like most natural product extraction processes), thermodynamic calculations can be very complicated. An alternative to performing highly complicated thermodynamic calculations consists on performing experimental designs (Schutz, 2007).

Experimental design gives the possibility to evaluate interactions between factors, in this case pressure and temperature, while limiting the number of

experiments. Different types of experimental designs have been used to optimize SFE extraction conditions; among them response surface methodology (RSM). RSM was first introduced by Box and Wilson (1951). The goal of RSM is to estimate a second-degree polynomial model that describes the response surface obtained in the experimental design. The higher R^2 of the model, the better it can predict future results and optimize the process. Although the extraction yield can be selected as the response variable, the particular composition of the extracts can also be optimized. For example, the use of RSM allowed the simultaneous graphical optimization of the extraction temperature, pressure, and time of different natural products such as passiflora seed oil and algal fatty acids (Sajilata, et al., 2008). In the extraction of passiflora seed oil, 14 experiments plus 6 replicates in the centre point were carried out to test 3 variables at 5 levels (Liu, et al., 2009). Oil extraction yield was the variable to optimize. Results showed a second-order polynomial model with good coefficients of determination (R^2=0.94) in which the linear and quadratic coefficients of independent variables, temperature, pressure, and extraction time, the interactions between temperature and extraction time and pressure and extraction time had a significant effect on the oil yield. The model predicted that the optimum extraction yield would be obtained using the following conditions: extraction temperature, 56 °C; extraction pressure, 26 MPa; extraction time, 4 h. Under these conditions, the oil yield was 25.83%.

Phase equilibrium for ternary system

In this section is presented brief introduction on phase equilibrium of ternary system. The system of three components composed from supercritical fluid and two others, one high volatile component and one low volatile component. For ternary systems Gibbs's triangular diagrams are showing the phase behaviour at constant pressure and constant temperature. Each corner of the diagram corresponds to one pure substance. The ternary diagram of water, ethanol and carbon dioxide is given in figure 2.3 (Catchpole, et al., 2009).

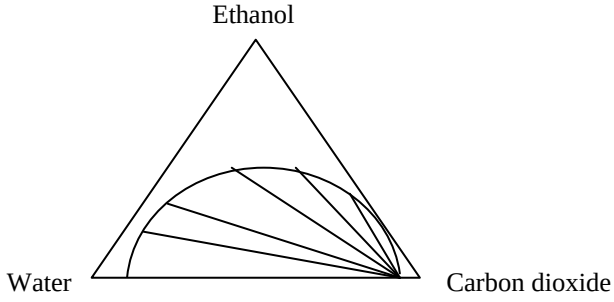


Figure 2.3. The Gibbs's triangular diagram at phase equilibrium for ternary mixture (CO_2-Ethanol-Water

The solid curve represents the critical line that limits the miscibility domain. The equilibrium lines show constant ethanol fractions in the vapor phase. At lower

pressures, both components are partially miscible with the SCF. The two-phase region is formed and the gas and liquid phases present different compositions, what is highly desired for every separation task. The coexisting phases are connected to each other by their respective tie lines. At higher pressures the high volatility components become completely miscible in the supercritical gas (Ksibi, 2004).

Supercritical fluid extraction

Supercritical Fluid Extraction (SFE) from solid particles consists one of the most widely used applications. SFE is a separation process where the substances are dissolved in a fluid under specific conditions above their critical parameters. The extraction is carried out through the continuous contact between a solid matrix and the solvent at high pressure. The solid substrate is loaded into the extractor, forming a fixed bed of particles. The supercritical fluid (SCF) continuously fed by a high-pressure pump at a fixed flow rate through extractor allowing the solubilisation of the desired components. The desired components are then continuously extracted by the supercritical phase until the solid matrix is depleted. Substances soluble in SCF reach separator where the extracted substances precipitate by temperature and/or pressure changes. More separation stages are often used to achieve a partial fractionation of the extract (Revercchon, 1997).

SFE is commonly carried out considering two basic steps: (1) extraction of soluble substances from the matrix by the SCF and (2) separation of the extracted compounds from the supercritical solvent after the expansion.

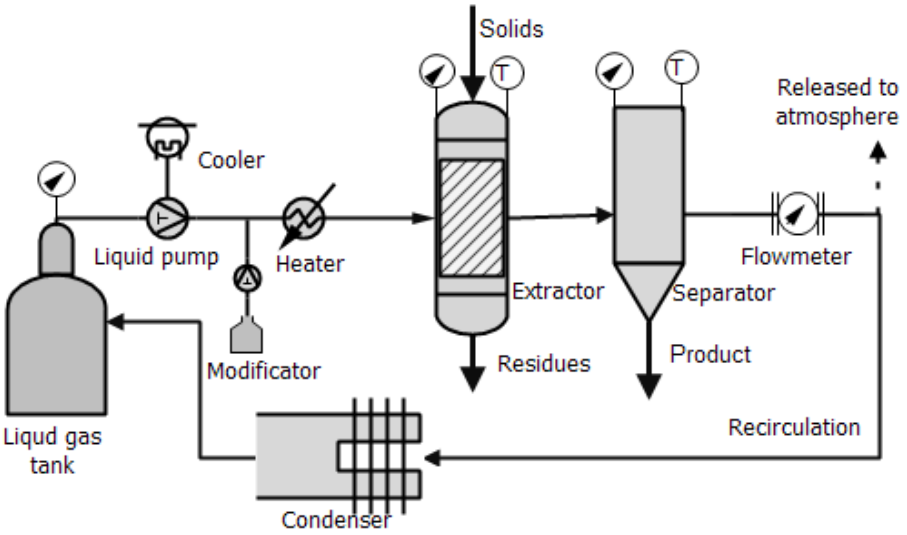


Figure 2.4. The scheme of SFE flow-sheet

The principal flow-sheet of SFE can be seen in Figure 2.4. Liquid gas is pressurised by CO_2 pump above it‘s critical point. Supercritical fluid under desired pressure and temperature conditions is fed at constant flow through the fixed bed solid material loaded in the extractor. The extraction cell is thermostated to obtain

appropriate extraction conditions. The solvent with dissolved solute flows to the separator where solute separates from the solvent by means of an increase in temperature or pressure reduction. Performing extraction process at smaller or analytical scale the gas phase of the solvent can be released to the atmosphere. If extraction is performed in higher scale and the gas consumption is high, gas can be recycled, closing the solvent cycle thus avoiding lost of the solvent.

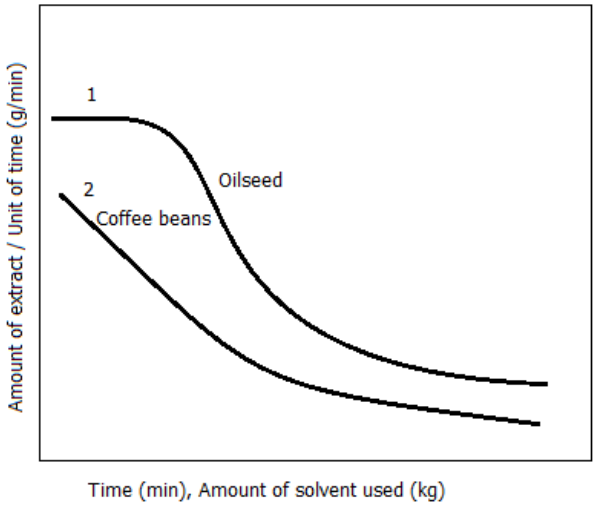


Figure 2.5. Extraction rate of oilseed and coffee beans under similar operational conditions (adapted from Brunner (1994))

A typical extraction process or overall extraction curve (OEC) can be divided into three periods (Meireles & Angela, 2007):

1. A constant extraction rate period (CERP), is characterized by the following kinetic parameters: the mass transfer rate of the solute contained in the surface of the particles, that is, easily accessible, the duration of the CERP, the yield during the CERP fluid phase at the extractor vessel outlet and mass ratio of solute in the fluid phase at the extractor vessel outlet. The mass transfer in this step is controlled by convection.

2. A falling extraction rate period (FERP), in which most of the easily accessible solute has been extracted and mass transfer starts to be controlled by diffusion.

3. A diffusion controlled rate period (DCRP), in which the easily extractable solute has been completely removed and the extraction process is controlled by the diffusion of the solvent inside the particles and the diffusion of solute + solvent to the surface.

The course of extraction from solid material can proceed various ways (Fig. 2.5). Curve 1 (Fig. 2.5) represents constant initial extraction rate from the solid matrix per unit of time. In the beginning of the extraction, mass transfer from oilseed is constant because solute is easily accessible for the solvent. After the initial period, during FERP, the decrease of solute in the solvent can be due to an additional

transport resistance, caused by the depletion of the solute in the solid matrix near the interface. The extraction rate with a low initial extraction concentration is represented by curve 2 (Fig. 2.5). Solute in the solid matrix is not readily accessible since beginning of extraction, mass transfer from coffee beans constant during all extraction period.

Extraction is always followed by another separation process where the extracted solute is separated from the solvent.

Another important aspect in supercritical extraction relates to solvent/solute interactions. Normally the interactions between the solid and the solute determine the ease of extraction, i.e., the strength of the adsorption isotherm is determined by interactions between the adsorbent and the adsorbate. However, when supercritical fluids are used, interactions between the solvent and the solute affect the adsorption characteristics due to large negative partial molar volumes and partial molar enthalpies in supercritical fluids (Montanes, et al., 2008).

SFE selectivity can be described by considering several parameters that should be optimised. Selection of the operating conditions depends on the specific compound or compound family to be extracted. The most relevant process parameter is the extraction pressure that can be used to tune the selectivity of the SCF. Working with solid materials, parameters as particle size, shape and porosity of the solid matrix, duration of the process and CO_2 flow rate are of crucial importance since they have direct effects on the thermodynamics (solubility) and kinetics (mass transfer resistance) of the extraction. Increasing mass transfer area of solid matrix, increase extraction rate, thus reduce the length of the solvent diffusion in the particle and decrease mass transfer resistance. However too small particles must be avoided, because can cause channelling inside the extraction bed, also can cause loss of volatile during the grinding. Generally, particles with mean diameters ranging between 0.25 and 2.0 mm are used. Increasing the amount of SC-CO_2 feed to the extraction vessel increase process rate. Solvent flow rate must be high enough to provide a good extraction yield in short time, but contact time among solvent and solutes should be sufficient. In general it is common to use solvent-to-feed ratios around 25–100:1 for analytical and 5–15:1 for large-scale processes.

The separation or fractionation of liquid mixture into fractions is another process of SC-CO_2. SC-CO_2 feed along the column from the bottom to the top, whereas liquid solution flows counter current. Operating parameters, as in the extraction of solids, is based on the different solubilities of the liquids. In order to extract all compounds in the mixture pressure and temperature have to be accurately chosen. The difference in density between the liquid and SC-CO_2 is used as a criterion to find the conditions of maximum selectivity. SCF density has to be lower than the density of the liquid mixture.

Supercritical antisolvent extraction (SAE) is a form of liquid extraction process. The scope of the process is recovery of target solid compounds from liquid mixture. The SC-CO_2 is mixing with liquid mixture in a pressurised precipitation vessel. According to selected conditions in precipitation vessel, compounds not soluble in SCF precipitate at the bottom of precipitation vessel and compounds mixture with SCF flow downstream to separator where they are recovered due to reduced pressure.

A schematic of the apparatus is shown in figure 2.6. The essence of this process is to produce very large liquid surface due to faster solubilisation of the liquid phase in supercritical media. A limitation of this process is the possible formation of a ternary mixture.

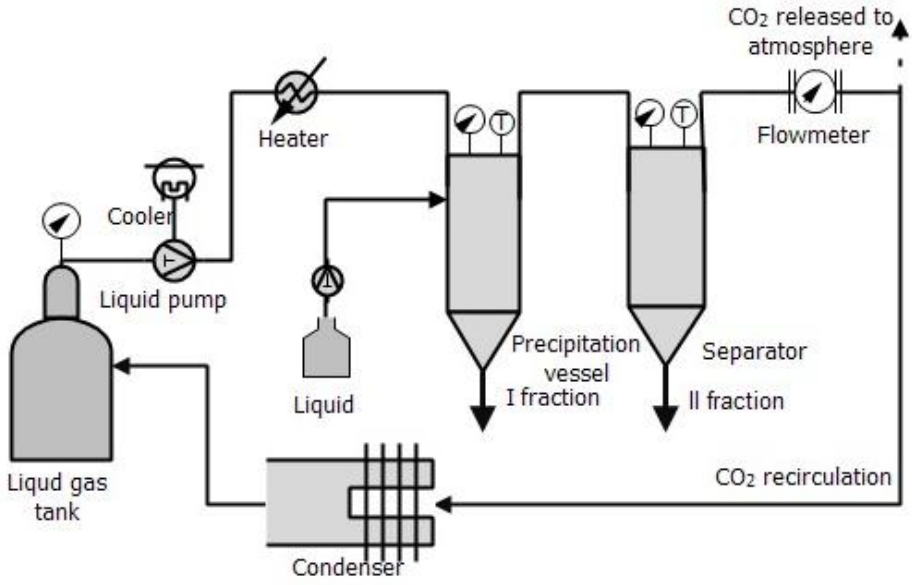


Figure 2.6. Schema of SAE

Modelling of Supercritical fluid extraction

Mathematical modelling of SFE gives understanding about general behaviour of extraction phenomena for given components. Phase equilibrium data such as solubility, distribution coefficient and selectivity in modelling of SFE is of great importance. The equilibrium may exist if desired substance is largely available on the surface of particle or near the surface and depends. Phase equilibrium depends on extraction pressure, temperature, on the composition of solute, solvent, and matrix. When the solute concentration in solid phase is high, like that of vegetable oil in seed, the fluid-phase equilibrium concentration is independent of matrix and equal to solubility. When the initial solute concentration in the plant is low, which is typical for essential oils, the equilibrium is usually controlled by solute–matrix interaction (Revercchon, 1997).

Process parameters, such as equipment dimensions, solvent flow rates and particle diameters must be carefully evaluated before predicting and scaling up procedures from analytical to pilot and industrial scales (Sovová, 2005).

Several aspects have to be considering before modelling SFE (Reverchon & de Marco, 2007):

1. *Solid material structure:* analysis of the material structure is necessary to form a view before modelling. For example, seeds are essentially formed by specialized structures that operate as small recipients containing the oil.

2. *Location of the compounds to be extracted:* The distribution of the solute within the solid substrate may be very different. The extractable substances may be free on the surface of the solid material or inside the structure of the material itself.

3. *Interactions of solutes with the solid matrix:* Depending on the interactions between the compounds and the solid structure, different equilibrium may be involved. The material can be adsorbed on the outer surface or inside the solid structure.

4. *Broken-intact cell structures:* Part of the compounds to be extracted maybe near the surface of the structure due to cell breaking during grinding. Moreover, membranes modifications may occur due to drying, freeing part of the soluble material.

5. *Shape of particles:* Particles may be spherical, plate like, or other shapes as a result of the original shape of the material (for example, leaves) and of the grinding process. Their shape can influence the diffusion path of the supercritical solvent.

Three different approaches for modelling of SFE has been proposed: based on heat and mass transfer (Reverchon, et al., 1993), as an expanded liquid involving semi-empirical correlations (Nguyen, et al., 1991) and differential mass balances integration (Roy, et al., 1996).

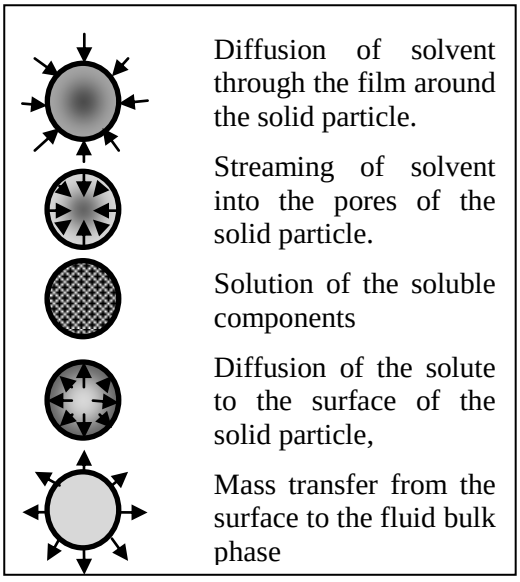


Figure 2.7. Steps of supercritical fluid extraction

Mass transfer resistances, in general, may be of two types: external or internal (Sovova, 1994). When there is free oil on the surface of fractured cells the pretreated substrate, the extraction rate is determined by the external mass transfer mechanisms. When oil is localized in undamaged cells and/or partially damaged cells, the extraction rate is determined by the internal mass transfer mechanisms. During the extraction internal diffusion is modelled using either effective diffusion coefficient

or solid-phase mass transfer coefficient. Two regions are distinguished in the particle: close to the surface there is a region of broken cells whose walls have been damaged by mechanical pre-treatment and other region particle core which contains intact cells. Mass transfer resistance from both regions is very different because the initial diffusion rate from broken cells is fast followed by much slower extraction from intact cells (Reverchon, et al., 2000).

Table 2.4. General model for SFE of solid matrixes in a packed bed

Differential mass transfer in the bulk fluid phase:			
	$\frac{\partial C_f}{\partial t} + u\frac{\partial C_f}{\partial z} - D_L\frac{\partial^2 C_f}{\partial z^2} = \frac{3}{R}\frac{1-\varepsilon}{\varepsilon}J$		(2.1)
Initial conditions:	$C_f = 0$	(at $t = 0$, for all z)	(2.2)
Boundary conditions:	$D_L\frac{\partial C_f}{\partial z} = uC_f$	(at $z = 0$, for all t)	(2.3)
	$\frac{\partial C_f}{\partial z} = 0$	(at $z = L$, for all t)	(2.4)
Differential mass transfer in the solid phase:			
	$\frac{\partial C_p}{\partial t} + \frac{\rho_p}{\varepsilon_p}\frac{\partial C_s}{\partial t} = \frac{D_e}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C_p}{\partial r}\right)$		(2.5)
Initial conditions:	$C_p = C_{p0}$	(at t = 0, for all r and z)	(2.6)
	$C_s = C_{s0}$	(at t = 0, for all r and z)	(2.7)
Boundary conditions:	$\frac{\partial C_p}{\partial r} = 0$	(at r = 0, for all z and t)	(2.8)
	$\frac{\partial C_s}{\partial r} = 0$	(at r = 0, for all z and t)	(2.9)
	$\varepsilon_p D_e\frac{\partial C_p}{\partial r} = -J$	(at r = R, for all z and t)	(2.10)
Definitions:			
Mass Transfer rate:	$J = k_f\,(C_p\|_R - C_f)$		(2.11)
Equilibrium relationship:	$C_s = f\,(C_p)$		(2.12)

According to Sovová (1994) model, the charge of column is homogenous and isotropic, and the soluble material is evenly dissolved in the raw material. Along the column the particle size distribution, temperature, and pressure are constant, and pressure drop is negligible. The inlet solvent is solute free. During the grinding process the cells on the surface will be crushed, and *q* fraction of the soluble components becomes free on the surface and can be extracted by simple dissolution, which is characterized by the first stage of the yield curve. The other part of the soluble components, (1-*q*) fraction, remains inside the particles and can only reach the surface by diffusion. The Sovová model divides the extraction process into steps shown in Figure 2.7. Any of these steps can be the one that determines the velocity

of the whole process. The mass transfer from solid matrix to surface is determined by inner mass transfer coefficient (k_s), while from surface to fluid phase by fluid mass transfer coefficient (k_f).

Mathematical models, based on mass balance, allow the calculation of yield in function of time and/or the determination of the remainder concentration in function of the extractor height in a certain extraction time.

Akgerman and Madras (1994) proposed general mass transfer model which is based on internal structure of a solid substrate particles, summarized in Table 2.4. The following assumptions are made: the solid particles are treated as spherical, porous and homogenous; interstitial velocity of the fluid is constant, bed porosity (ε) remains unchanged; physical properties of the SCF and substrate in the bed are constant; pressure losses and temperature gradients are negligible within the bed; the solute is subjected to axial dispersion.

Solute in the SCF flows along the bed (diffusion coefficient D_L) from high concentration zones to low concentration zones.

In the solid matrix two solute fractions can be found, the solute is adsorbed into the solid (concentration C_s), and the solute dissolved in the fluid phase within the pores (concentration C_p). Initial concentrations C_{s0} and C_{p0} are determined by the total initial solute concentration (C_0) and equilibrium between the two phases, Equation 2.13:

$$C_{s0} = f(C_{p0}) \quad (2.13)$$

$$\varepsilon_p C_{p0} + \rho_p C_{s0} = C_0 \quad (2.14)$$

By assuming the considerations above, solute concentrations in the solid matrix (C_s and C_p) depend on radial position within the particle (r), axial position (z), and extraction time (t); whereas the total solute concentration in the SCF (C_f) depends only on z and t.

Del Valle and de La Fuente (2006) reviewed models based on mass transfer of vegetable oils to facilitate the scale-up of laboratory data for industrial scale purpose. Seeking to scale-up SFE process mass transfer models for the packed bed should be based on mass balance.

Westerman et al. (2006) discussed mathematical modelling of the solubility and the extraction process. The Chrastil correlation (Eq. 2.15) and the Del-Valle and Aguilera modification (Eq. 2.16) were used for the solubility modelling of squalene and total extract oil of amaranth.

$$\ln S = k \ln \rho + \left(\frac{a}{T} + b\right) \quad 2.15$$

$$\ln S = k \ln \rho + \left(\frac{a}{T} + b + c\right) \quad 2.16$$

Where, S is the solubility for a given temperature, ρ - solvent density, k is straight line slope, T – temperature and a, b, c are independent terms.

The Del-Valle and Aguilera modification to the Chrastil model is to introduce a further temperature dependency to the secondary solubility constant and to introduce a third independent *c* term. The effect of this addition is to maximise the temperature

rather than density dependency of the model. They determined that the extra dependency parameters introduced by Del-Valle and Aguilera have a greater impact on pseudo-components or solutes entrapped within a particle matrix such as the amaranth seed. In contrast to squalene, where the solute is a true single component, the Amaranth, solutes are a complex mixture of compounds and treated as a pseudo-component.

Results from the extraction experiments were compared against the Characteristic Time Model (CT) (Eq. 2.17).

$$DE_t = DE_\infty \left[1 - exp\left(-\frac{t}{t_m}\right)\right] \qquad 2.17$$

Where, DE - degree of extraction, t_m – diffusion time.

The CT model successfully describes the oil extraction from amaranth. Diffusion coefficient values were calculated from the Shrinking Core Model. Westerman et al. (2006) concluded that the CT model shows an excellent fit to experimental data. This model could be easily adapted to scale up process from pilot scale to industrial scale due to its simplicity. At very low solvent densities the CT model is less able to accurately predict the course of the extraction.

A typical extraction profile has two distinct phases, which correspond to the internal (solubility limited) and external (mass transfer limited) resistances. In the case of amaranth the CT model the external resistance type profile predominates.

Scale-up of supercritical fluid extraction

Supercritical fluid extraction (SFE) processes can be scaled-up from lab-scale or pilot-scale results according to an industrial. There are many industrial scale equipments working with supercritical fluids around the world in many area of industry. There are some basic rules handling supercritical processes, safety is the most important and must be taken into account at any step of equipment design, installation, operation and maintenance. Mechanical hazards as well as thermodynamic, chemical and biological hazardous must be conducted for any case.

Before commercializing supercritical fluid extraction following question must be answered (Meireles & Angela, 2007):

1. What is the best process for obtaining the desired extract?
2. For a given extract with specified functional properties, would SFE be a good choice?
3. If SFE is an alternative, what are the pressure and temperature of extraction?
4. At this pressure and temperature, what is the process yield?
5. How long does it take to obtain such yield?

Analytical scale experiments are very important in order to adjust optimal extraction conditions through a scanning of different extraction conditions as: pressure, temperature, solvent ratios and composition.

Solubility and diffusion are the mechanism which control scale-up of extraction. For instance, in the case of solubility, the access to the extract in the matrix is easy. Although extraction limited by diffusion is more static and complicated, especially the internal diffusion.

Depending on the complexity and on the kinetic limitations of the extraction, different methods are available to design the production unit:

The easiest method to scale-up experimental data is to keep one or both of the ratios U/F and S/F constant, where U is the solvent flow rate (in kg/h), F is the feed mass in the extractor (kg), and M is the solvent mass required for the extraction (kg). When both diffusion and solubility control the extraction, both ratios U/F and S/F shall be maintained constant. This method does not take into account several important factors (internal diffusion, axial mixing, etc.).

A refined method integrating these factors requires a numerical simulation that can estimate any configuration and permit industrial-plant optimization (Clavier & Perrut, 2004).

Advantages and drawbacks of supercritical CO_2 (SC- CO_2)

Advantages:

- Favorable critical values (T_c - 31.1 ºC; P_c – 7.2 MPa; d_c - 0.47 gcm^{-3});
- GRAS (to overcome legal restrictions applied to organic solvents and to allowed residues in products for human use);
- Ecological (CO_2 from the atmosphere, is not generated in the process);
- It is possible to obtain natural products with very low levels (or even without) residues using SC-CO2 at low temperatures;
- Cheap;
- CO_2 + ethanol (GRAS) can be employed to extract more polar compounds.

Drawbacks:

- Low affinity for medium and high polarity compounds.
- The main drawback of SFE for essential oils is that investment costs are higher than those for traditional atmospheric pressure extraction techniques and selectivity is low. Fractional separation of the extracts is one of the methods that can be useful in improving SFE selectivity.

To widen the application range of this technique is the study of new methods to decrease analyte polarity to make them more soluble in non-polar supercritical fluids. In this sense, chemical in situ derivatization has been applied to improve the selectivity of the extraction towards a specific group of compounds (Hawthorne, et al., 1992).

The solvent power of SC-CO_2 can be summarized by a few rules:

- It dissolves non-polar or slightly polar compounds.
- The solvent power for low molecular weight compounds is high and decreases with increasing molecular weight.
- SC-CO_2 has high affinity with oxygenated organic compounds of medium molecular weight.
- Free fatty acids and their glycerides exhibit low solubility's.

- Pigments are even less soluble.
- Water has a low solubility (<0.5% w/w) at temperatures below 100 °C.
- Proteins, polysaccharides, sugars and mineral salts are insoluble.

SC-CO_2 is capable of separating compounds that are less volatile, have a higher molecular weight and/or are more polar as pressure increases (Brunner, 2005).

SFE applications for natural materials

Since the end of XX century (in the late 1980's and early 1990's) SFE became an alternative extraction technique in many fields ((Mc Huahg & Krukonis, 1994). SFE process is fast growing technology since it can overcome many disadvantages comparing with conventional extraction technologies obtaining high quality "natural" products. Supercritical fluids have been applied to many industrial areas: mass-transfer processes, reactive systems, phase-transition processes, materials-related processes, nanostructured materials, impregnation and cleaning, particle formation, multistage countercurrent separation, coating, biomass gasification and supercritical water oxidation. Many applications are already at industrial capacity, whereas some remain under development. The supercritical carbon dioxide (SC-CO_2) is the most applied supercritical solvent for food applications, especially extraction and fractionation of natural products from solid matrix. Mukhopadhyay (2000) described applications of SFE in processing of natural and food products: Decaffeination of coffee and tea, spice extraction (oil and oleoresin), deodorization of oil and fats, extraction of vegetable oils from flaked seeds and grains, flavours, fragrances, aromas and perfumes, hops extraction from bitter, extraction of herbal medicines, stabilization of fruit juices, lanolin from wool, de-oiling of fast foods, de-cholesterolization of egg yolk and animal tissues, antioxidants from plant materials, food colours from botanicals, de-nicotinization of tobacco, cleaning of oils. There are a number of papers concerning supercritical fluid application for the extraction of various biological materials, Mendiola et al. (2013) reviewed latest published works in which the use of this extraction technique is described for recovering bioactive compounds from various natural raw materials.

Extraction of Essential Oils and Edible Oils. Essential oil is a volatile small portion of a plant material which consists mainly of hydrocarbon and oxygenated terpenes and hydrocarbon and oxygenated sesquiterpenes. Essential oil can be from seeds, roots, flowers, herbs and leaves. Reverchon (1997) reviewed solubility data, extraction, fractionation and modelling aspects of essential oil isolation with SC-CO_2. Essential oil can be optimally isolated operating at mild pressures (from 9 to 10 MPa) and temperatures (from 40 to 50 °C) because, at these process conditions, all the essential oil components are largely soluble in SC-CO_2 (Raeissi & Peters, 2001) (Akgün, et al., 1999). During essential oil extraction waxes and essential oil are coextracted at temperatures above +5°C, therefore it is necessary to maintain low temperatures (from –5 to +5°C), at these temperatures waxes are practically insoluble in CO_2, whereas terpenic compounds are completely miscible in liquid CO_2 (Reverchon, et al., 1993). The SFE has proved effective in the separation of

essential oils and their derivatives for use in the food, cosmetics, pharmaceutical and other related industries, producing high-quality essential oils with commercially more satisfactory composition than obtained with conventional hydro-distillation (Diaz-Maroto, et al., 2002). To increase SCF selectivity and avoid the coextraction of compounds, few concepts of SFE could be applied: fractional SFE fractionation, multistage extraction and cosolvent addition. Essential oil extraction and fractionation using SC-CO_2 was widely discussed by Reverchon and de Marco (2007).

Carbon dioxide is an ideal SCF for edible oils and their components extraction. SFE of lipids has received attention as an alternative to organic solvent extraction and has been shown to be an ideal method for extracting certain lipids (Garcia, et al., 1996). Although triacylglycerides are only fairly soluble in SC-CO_2, the advantages of edible oil processing with SC-CO_2 include low processing temperatures; minimal degradation of the minor thermolabile compounds; solvent free products; minimizes undesirable oxidation reactions. Edible oils isolated from certain natural materials contain various health beneficial, sensitive bioactive components such as sterols, tocols (tocopherols and tocotrienols), carotenoids, and polyunsaturated fatty acids (PUFAs), squalene. Such oils include nut oils (almond, hazelnut, peanut, pecan, pistachio, and walnut), seed oils (borage, flax, evening primrose, grape, pumpkin, and rosehip), cereal oils (amaranth, rice bran, and oat and wheat germ), and fruit and vegetable oils (buriti fruit, carrot, olive, and tomato) (Temelli, et al., 2007).

Aleksovski et al. (1998) compared SC-CO_2 extraction with conventional extraction of grape seeds. The comparison of the two applied methods shows that supercritical extraction is an efficient method by which natural oil can be obtained. The use of carbon dioxide offers certain advantages concerning the quality of the extracted oil and the efficiency of the process. Maness et al. (1995) also obtained similar results to those with conventional methods of pecan oil with SC-CO_2. Extraction of poppy seed oil with supercritical carbon dioxide (SC-CO2) was performed and the effect of extraction conditions on oil solubility and yield as well as oil composition was evaluated by Bozan and Temelli, (2003). Kraujalis and Venskutonis (2013) optimised amaranth seed oil yield using SC-CO_2 extraction. The composition of fatty acids and squalene content were analysed by chromatographic methods. The solubility of amaranth seed oil increased with temperature at high pressures, however it decreased with increasing temperature at lower pressures. Similar interaction of peanut oil in SC-CO_2 observed Goodrum and Kilgo (1987). SCE-CO_2 was successfully applied as a separation and fractionation technique for reducing free fatty acid content, minimizing phytosterols loss in rice bran oil (Dunford & King, 2000). Montanari (1996) increased selectivity of phospholipids extraction from soybean flakes mixing co-solvent with SC-CO_2. Rozzi and Singh (2002) extensively reviewed fatty acids extraction studies from various natural sources using SC-CO_2. Zaidul et al. (2006) reported that SC–CO_2 can be applied to fractionate palm kernel oil to decrease C8–C14 levels and concentrate C16–C18:2 fatty acid constituents. Pressure from 34.5 to 48.3 MPa at 80 °C were found to be effective in the fractionation of medium (C8–C14) and longer (C16–C18:2) chain fatty acid constituents. Markom et al. (2001) studied the fractionation of crude palm

oil using SC–CO_2 at 40–60 °C and 14–20 MPa. They observed that the solubility of crude palm oil was generally low at temperature up to 60 °C and pressure up to 20 MPa. The solubility of fatty acids in SC–CO_2 extraction depends on the length of the hydrocarbon chain and the presence of functional groups, as well as on the effect of the extraction parameters, such as pressure and temperature (Stahl, et al., 1988).

Since lipids are soluble in SC-CO_2 it can be applied to clean or remove undesirable fats from food.

Extraction of antioxidants. The most studied antioxidants extractable from vegetal biomass by SFE with CO_2 are phenolics, terpenoids, carotenoids, and tocopherols. SC-CO_2 is a very attractive method for extraction of nonpolar bioactive thermo labile compounds as tocols, terpenoids and carotenoids. Tocopherols also known as vitamin E possess strong antioxidative activity and can protect unsaturated lipids in vivo. They are also important nutritional components acting as biological kidnappers of free radicals and participating in prevention of various diseases (Brigelius-Flohé, et al., 2002). Eight forms of this vitamin E can be found under natural conditions, namely: α-, β-, γ-, and δ-T (tocopherols), as well as α-, β-, γ- and δ-T3 (tocotrienols). However the latest North American guidelines consider α-tocopherol to be the only biologically active form of vitamin E (Anon, 2000). Vitamin E has been widely applied in the field of food, medicine and cosmetic.

Commercially natural tocopherols were concentrated from methyl esterified deodorizer distillate of a by product of the edible oil refining process. Final pressure of 20 MPa resulted in relatively high average tocopherol content (> 50%) and tocopherol recovery (about 80%) (Fang, et al., 2007). Terpenoids, also known as *isoprenoids,* are secondary plant metabolites accounting for the largest family of natural compounds. Terpenoid compounds (monoterpenes, sesquiterpenes, and diterpenes) are the main components of essential oils. Carotenoids are synthesized by plants, photosynthetic organisms, some bacteria, yeasts and molds. They are classified as carotenes (α- and β-carotene, lycopene), composed only of carbon and hydrogen atoms, or xanthophylls (zeaxanthin, lutein, α- and β-cryptoxanthin, canthaxanthin, astaxanthin), with at least one oxygen atom. Some carotenoids (α- and β-carotene, β-cryptoxanthin) are precursors of vitamin A and protect against chemical oxidative damage, several kinds of cancer degeneration (Stahl & Sies, 2003).

There are number of published works regarding the use of SFE for the extraction of bioactive compounds from plants (Mendiola, et al., 2013). Although carbon dioxide is restricted by its inadequate solvating power for highly polar analytes, there are overcomes by using an appropriate modifier. SFE modifiers such as ethanol are introduced at the levels of 1–10%; large modifier concentrations (10 – 50%) are also of interest in some applications. Optimization of the operating conditions such as pressure, temperature, percentage of modifier, and the extraction time are generally considered as the most important factors for good recoveries (Krishnaiah, et al., 2007). The results of comparison of different extraction procedures for isolation of the antioxidant 5.8 dihydroxycoumarin from sweet grass (*Hierochloë odorata*) were done by Grigonis et al., (2005). Two-step supercritical fluid extractions were performed. In the first series the herb was pre-extracted with

30 L of pure CO_2 and afterwards the residue was re-extracted with 60 L of CO_2 + 20% of ethanol at 35 MPa and 40 °C for both steps. Two-step SFE extraction gave an antioxidant mixture with almost 22.5% antioxidants (both 1 and 2).

Recently supercritical fluid antisolvent fractionation was used to obtain antioxidant compounds, mainly carnosic acid (CA), from high viscous oleoresins derived from dried rosemary leaves (Rosmarinus officinalis) extracted with ethanol (Visentín, et al., 2011). Experiments were conducted at 50 °C and six different pressures in the first separation vessel, ranging from 15 to 40 MPa. The highest yield (33% in average) was achieved at 30 MPa.

Bimakr, et al., (2009) extracted flavonoid compounds from leaves of spearmint (*Mentha spicata L.*) using supercritical carbon dioxide (SC-CO_2) extraction. The results indicated that pressure, temperature and dynamic extraction time had significant effect on the extraction yield. The highest extraction yield (60.57 mg/g) was obtained at 20MPa, 60 °C and 60 min.

Maróstica et al., (2010) updated overview on the principal applications of SFE in recovery of bioactive phenolics emphasizing the effects of temperature, pressure, addition of co-solvents and time on the extraction yield of these compounds and their antioxidant activities for a possible applications in food, cosmetic or pharmaceutical industries.

In a recent paper by Klejdus et al. (2009), a new methodology was proposed to isolate natural bioactive substances in biological matrices based on the use of a new SPE/SFE hybrid extraction. A new extractor device was designed, allowing the insertion of the SPE cartridge into the cell; after careful optimization of the extraction and elution conditions, it was possible to recover 13 phenolic compounds from different cyanobacteria and microalgae.

Other SFE applications in food industry. Supercritical Carbon dioxide proved to be highly selective for alkaloids such as caffeine, morphine, emetine, pilocarpine. Recent investigations have demonstrated the potential exploration of solvent and anti-solvent properties of carbon dioxide in the recovery of alkaloids such as theophylline, theobromine and pilocarpine, among others (Mohameda & Mansoori, 2002).

Another new commercial application of SFE is cleaning of rice. The rice cleaned by supercritical CO_2 has advantages over the conventional product: Pesticides and heavy metals are removed, germs and insect eggs are destroyed, waxy layer and fatty acids are removed. Cooking time is shortened by 30%, and the shelf life of the bagged rice is extended and quality enhanced (Brunner, 2010).

Application of very high pressures (>100 MPa) in commercial extraction may be of interest for the processing of pharmaceutical compounds that are difficult to dissolve (Brunner, 2010).

There are some examples of the many developments with SFE, representing all fields where this technology may be applied (Raventos, et al., 2002). Sowbhagya and Chitra (2010) reviewed the used of enzymes- assisted extraction with liquids and supercritical phases. Its main application has been in plant matrices to break cell walls increasing in extraction efficiency and/or selectivity of the extraction process. Van Iersel et al. (2010) studied sample preparation improvement using ultrasound

irradiation, ultrasound assisted extraction combination with SFE has gained interest due to the higher extraction yields that can be obtained in shorter times. Ultrasound irradiation of high-pressure fluid leads to improvements in mass and heat transfer in high-pressure fluid due to the creation of an extremely fast, local phase separation.

SFE process development

Particle size distribution plays a crucial role in the amaranth extraction procedure by SFE. Although to small particles size of oily samples may lead to oil lost or oxidation during milling because of strong mechanical impact. It is important to note that the moisture content within 0% to 10% had little influence on oil yields at 40 ◦C and 25 MPa (He and others 2003). Amaranth seeds may be processed for consumption in different ways and processing parameters may affect the oil. Fractional separation of the extracts is useful in improving SFE selectivity. Using fractionation is possible to separate in two or more fractions of different composition by setting appropriate pressure and temperature in the separators (Wang, et al., 2004). In order to separate several compound families from the same matrix that possess different solubility's in SCF multistage extraction could be applied. Multistep extraction involves varying pressure or temperature conditions in each extraction step (Gaspar, et al., 2003).

2.2. Others pressurised fluid techniques

Principles

In recent years, several novel extraction techniques have been developed seeking to overcome the limitations of the conventional methods. In general, these alternative extraction techniques allow a more efficient isolation of the analyte from the matrix by improving the contact of the target compound(s) with the extraction solvent which allows a reduction of both the extraction time and the organic solvent consumption and increases sample throughput. Enhanced extraction efficiency can be achieved by using solvents at high pressures and temperatures, as in pressurised liquid extraction (PLE) also known as accelerated solvent extraction (ASE) or the closely related subcritical water extraction (SWE) also known as pressurised hot water extraction (PHWE). PLE as well as SWE methods are developed at elevated pressure and temperature conditions operating above liquids normal boiling point. Solvents during extraction are below their critical values, so the solvent maintain the same liquid state during all extraction. Higher temperature, increase diffusion rate, solubility and mass transfer of analyte and decrease the viscosity and surface tension of the solvent. These properties improve better contact between analyte and the solvent also enhance extraction within shorter time (20 min is enough for quantitative extraction) and with less solvent consumption (on average 10-50 ml) comparing with conventional extraction. Moreover, extraction procedure becomes versatile through tuning different operating parameters (e.g. temperature, pressure, time, extraction cycles and solvent). PLE and SWE are performed in static or dynamic mode. Most of extractions were performed in static mode followed by dynamic flush with organic solvent after static mode (Ramos, et al., 2002). The main

different between PLE and SWE is the nature of solvent used for the extraction. The variables affecting the extraction process, such as the nature and temperature of the extraction solvent have profound effect on the efficiency of extraction. The solvent must be able to solubilise the target analyte while leaving the sample matrix intact. The polarity of the extraction solvent should closely match that of the target compounds (Mukhopadhyay, et al., 2006). Other variables as pressure have been reported to play no role other than to keep the extraction solvent liquid at the high-temperatures, because many solvents used in extractions boil at relatively low temperatures. Pressure also force solvent into the pores of the sample matrix, thus coming into close contact with analyte (Ramos, et al., 2000).

Figure 2.8 shows a basic scheme of the equipment used to perform compressed solvent extraction.

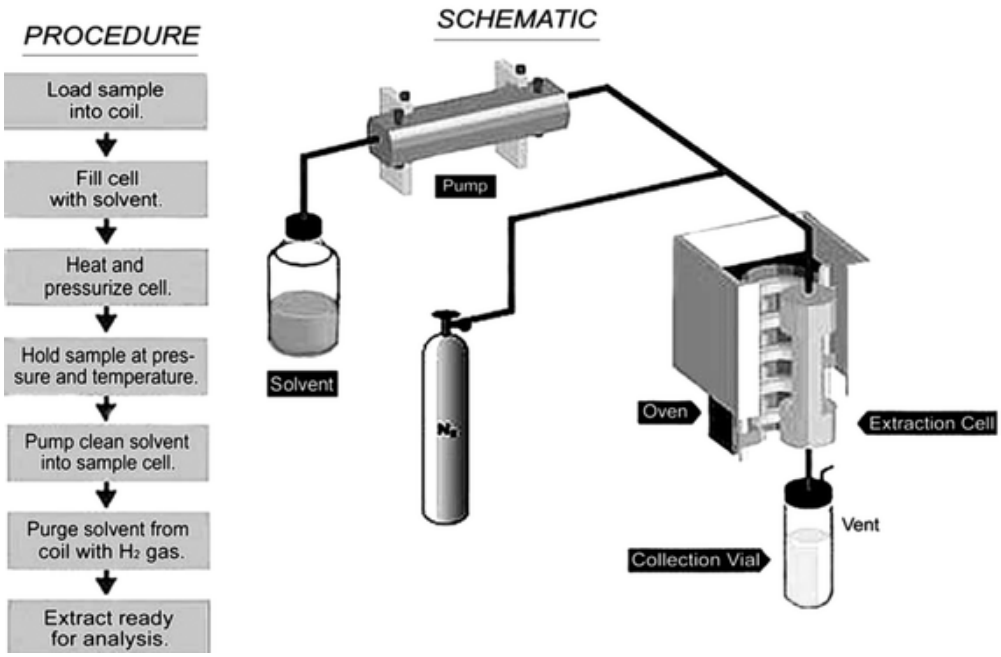


Figure 2.8. Compressed solvent extraction scheme (Wanekaya, et al., 2002)

SWE is particularly form of extraction, when as a solvent is using water above it‘s boiling point 100 °C and below it‘s critical temperature point 374 °C. While 10 MPa pressure is using to maintain water in a liquid state. Temperature is the main factor influencing water as a solvent selectivity and extraction efficiency. By varying in temperature physical and chemical properties of water change dramatically.

An increase in temperature:

- analyte diffusion (diffusivity of water at 25 °C is about 10 times lower than that of water at 200 °C);
- favours mass-transfer kinetics by disrupting intermolecular forces (i.e. Van der Waals forces, hydrogen bonds and dipole attractions);

- decreases the viscosity of water (enabling better penetration of matrix particles);
- decreases the surface tension (allowing the water to better wet the sample matrix);
- weaken hydrogen bonds, resulting in a lower dielectric constant (ϵ). Dielectric constant varies from 80 (at 25 °C very polar) to 27 (at 250 °C between those of methanol ($\epsilon = 33$) and ethanol ($\epsilon = 24$) at 25°C) at 20 MPa (Teo, et al., 2010).

Process development

Using PLE technique selectivity is the main target, to extract compounds of interest with little or no interfering coextracted compounds. To obtain better selectivity, is necessary to optimised extraction factors. Optimization of different extraction factors influence the yield or selectivity of the process. There are three techniques: choice of temperature, choice of solvent, and use of adsorbents in the sample cell. As already stated, temperature is critically important to the extraction procedure. The higher temperature of the extraction, results in worse selectivity. Lowering the temperature extraction will be more selective, but the recovery of analytes can diminish, unless the time is increased. Also high temperatures might have negative effects on the bioactivity of some thermo-labile compounds. During the extraction, formation of new components in the extracts, as a result of the different reactions, has to be considered (Plaza, et al., 2010). Selectivity possibly decreases using more polar solvents. To avoid that drawback is possible to use a series of solvents of increasing polarity to obtain fractionations with different polarity compounds (Kraujalis, et al., 2013). Solvents mixture as ethanol or methanol with water was used to isolate polar compounds obtaining better extract yield (Hossain, et al., 2011).

The influence of extraction time will directly depend on the PLE mode applied, static or dynamic.

Commercial instruments mostly are using static extractions, in which a certain volume of solvent, under the desired conditions of pressure and temperature, is maintained in contact with the sample for a given time. The efficiency of the extraction procedure could be increased using several consecutive static extraction cycles. For example, the yield of phenolic compounds from parsley was increased by using four consecutive extraction cycles comparing with one cycle (Luthria, 2008). However, in some applications, one extraction cycle is enough to extract the target compounds (Lee, et al., 2010).

Ezzell et al. (1996) reported the use of alumina sorbent in the sample cell to increase selectivity of PCBs from fish tissues. The adsorbent is loaded into the cell before the sample. Unwanted compounds are retained in the cell by the adsorbent. The capacity of the sorbent to retain lipids decrease if temperature or polarity of the solvent increase. Many others sorbents like silica, florisil, C18 resin could be used to retain lipids (Gentili, et al., 2004). Poerschmann and Carlson (2006) demonstrated the fractionation of lipid classes using sequential extractions. Very good separation

was obtained using combination of adsorbents in the cells, varying temperature, and solvent fractionation.

In recent year ionic liquids (ILs) have been revealed as new type of green solvent because which could be potentially applicable in PLE (e.g., high solvent power, high chemical and thermal stability, nonflammability and non-volatility) (Herrero, et al., 2013).

SWE as PLE are characterised by similar working mechanism, however SWE is highly relevant from an environmental point of view, since water is used as a solvent. One of the major limitations of SWE is the low water solubility of certain compounds. Static mode is also the most frequent method in which equilibrium is reached between the sample components and the water phase. Using dynamic extraction more pressurised hot water flows through extraction cell in the end obtaining bigger amount of diluted extract. That increase expenses to remove water from the solvent. A new online process (WEPO) has therefore been developed involving SWE plus drying the extracts in a single step (Ibáñez, et al., 2009).

Applications

PLE or SWE are regarded as environmentally friendly methods for extraction of bioactive substances from various natural samples also various chemicals or contaminants from food or environmental samples. The samples as plants are the most studied natural material for PLE and SWE. Many applications of PLE as well as SWE have been used for the bioactive compounds, especially antioxidants, extraction from plants, algea and other natural matrices (Herrero, et al., 2013). The wide range of articles have been published in a field covering environmental, food and drugs, agricultural applications (Turner & Ibañez, 2011). SWE have been applied to the determination of pesticides, contaminants, and insecticides in fruits, sediments, milk, soils (Kronholm, et al., 2007). Food and agricultural applications involves health-beneficial or bioactive compounds isolation from various plants and foods. Many of these compounds possess antioxidant properties. Many studies are based on experimental conditions optimisation for certain compounds or yield maximising (Wijngaard & Brunton, 2009). Terpenoids isolation has limited application because of scarce solubility in water even at elevated temperatures, these compounds are commonly extracted using PLE with 2-propanol or methanol/ethyl acetate/ light petroleum (1:1:1) at temperatures ranging from 40°C to 190°C (Breithaupt, 2004).

Hot liquid water (HLW) hydrolysis, under high pressure and temperature (160–240°C, 1–5 MPa), is recognized as one of the most promising methods, for example cellulose hydrolysis to ethanol for biofuels production, since the process does not need a chemical catalyst, and neither neutralization or detoxification are required after pretreatment (Zhuang, et al., 2009).

PLE with ethanol at 200 °C was shown to be the most appropriate process to obtain extracts with the highest antioxidant activity from rosemary compared to other pressurized extraction methods including SWE (Luthria, 2008).

In-cell enzyme pre-treatments prior to extraction might be a good option to increase the extraction yield of particular compounds. Enzymatic sample

pretreatment might be best suited to SWE. SWE also has been employed to separate dietary fibres from the residue obtained by supercritical CO2 of citrus, obtaining valuable compounds from citrus-fruit waste (Tanaka, et al., 2012).

2.3 Process optimisation methodology

Optimisation refers to improving the performance of the process to obtain the maximum response value. Response surface methodology (RSM) is a statistical technique, which is used to evaluate the effect of multiple factors and their interaction on one or more response variables. Central composite design (CCD) is the most popular form of RSM and it could be utilized in many fields of research to optimise various processing methods. CCD fit for sequential experimentation, is very efficient and provides much information on experiment variables effect. CCD requires fewer experimental runs comparing with full factorial design and enables to obtain statistically acceptable results (Tan, et al., 2009).

There are two main varieties of CCD: face-centered and rotatable (Fig. 2.9).

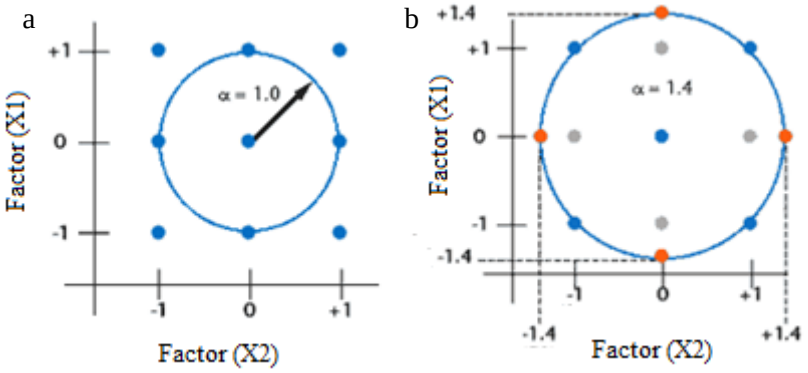


Figure 2.9. Two-variables face-centered (a) and rotatable (b) CCD

Values of variables are coded to lie at ±1 for the factorial points, 0 for the center points and ±β for the axial points (Table 2.5).

Table 2.5. Relationship between coded and actual values

Coded values	Actual values
-β	x_{min}
-1	$[(x_{max}+x_{min})/2]-[(x_{max}-x_{min})/2\alpha]$
0	$(x_{max}+x_{min})/2$
+1	$[(x_{max}+x_{min})/2]+[(x_{max}-x_{min})/2\alpha]$
+β	x_{max}

x_{max} and x_{min}=maximum and minimum values of x, respectively; $\alpha=2k^{/4}$; k=number of variables

In general response variable y can be expressed as:

$$y=f(x_1,x_2)+\varepsilon \qquad (2.18)$$

Where: x_1 and x_2 are independent variables, $f(x_1,x_2)$ represents surface and is called response surface, ε experimental error term.

First-order model or screening model involves linear effect and could be expressed as:

$$y=\beta_0+\beta_1 x_1+\beta_2 x_2+\beta_{12} x_1 x_2+\varepsilon \quad (2.19)$$

Where: β_0 is constant, x_{ij} independent variables

In order to determine a critical point, it is necessary to use second-order polynomial model (Eq. 2.20) (Bezerra, et al., 2008):

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2.20)$$

Where: β_{ij} - represents the coefficients of the quadratic parameter, ε – error terms, x_{ij} – independent variables, k number of variables.

RSM was employed to optimise SCE-CO_2 of oil from various materials (Ju, et al., 2010), (Mariod, et al., 2010). The quality of the polynomial model was expressed by the coefficient of determination, namely, R^2 and Adj-R^2. The statistical significance was verified with adequate precision ratio and by the F test.

Amaranth (*Amaranthus spp.)*

Amaranth seeds

Figure 2.10. Amaranth plants and seeds

2.4. Amaranth

Physiology and general characteristics

The word "amaranth" in Greek means "everlasting". Amaranth plant is fast growing pseudocereal that has promising potential as a nutritious subsidiary food crop. Amaranth was important food crop in the Aztec, Mayan, and Incan

civilizations. More than 35 years ago, there has been increasing interest in grain amaranth. Researchers, farmers and food processors have been studying amaranth on three areas: germplasm preservation and utilization, the development of improved lines, and outreach and information dispersal. A number of conferences for the promotion of the amaranth crop have been held in the end of 20th century (Kauffman & Weber, 1990).

The genus *Amaranthus* (family *Amaranthaceae*) includes approximately 60 species and could be consumed as a grain or a vegetable. There are some species commonly grown for a grain like *A. cruentus* L. (purple amaranth), *A. hypochondriacus* L. (prince's feather) and *A. caudatus* L. (love-lies-bleeding) also for ornamental, but *A. tricolor* (tampala) is grown primarily for the leaves (Fig. 2.10).

In Lithuania grow following varieties of amaranth: baltasis burnotis (Amaranthus albus), gulsčiasis burnotis (Amaranthus blitoides), uoginis burnotis (Amaranthus blitum), uodegotasis burnotis (Amaranthus caudatus), raibasis burnotis (Amaranthus cruentus), šiurkštusis burnotis (Amaranthus retroflexus), šluotinis burnotis (Amaranthus paniculatus). There are three genotypes of amaranth grown for agricultural purpose: 'Raudonukai', 'Rausvukai' and 'Geltonukai'. 'Geltonukai' or 'Rausvukai' mainly grown for grains and 'Raudonukai' are used mainly due to biomass and fodder for animals (Svirskis, 2003). Amaranth may grow up to 2 meters and produce yields comparable to rice or maize 2,500 kg/ha. Svirskis (2003) investigated some parameters of amaranth growing technology as sowing time, seed rate and row spacing. The highest yield was produced when amaranth had been sown in the middle of May, at a seed rate of 2–4 kg ha-1, with row spacing of 50 cm and thrashed dry after severe frosts (-3…-5°C).

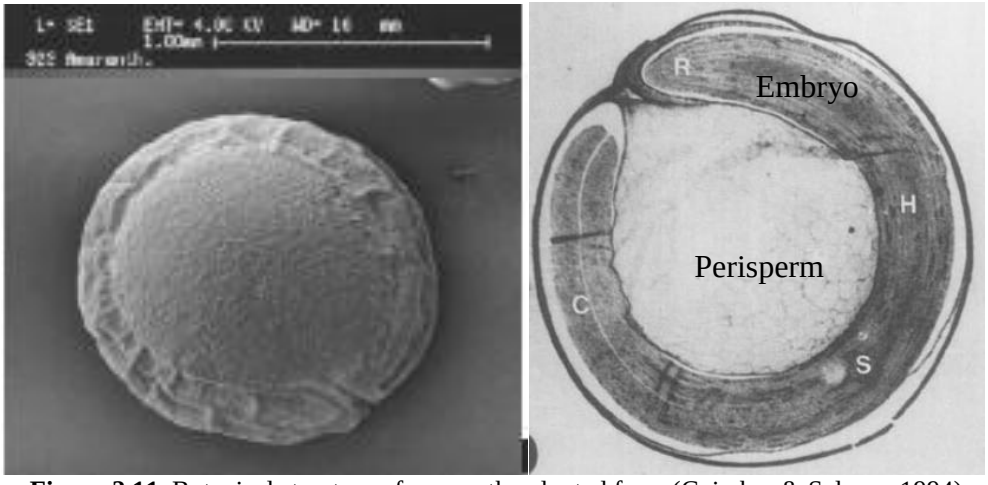


Figure 2.11. Botanical structure of amaranth, adopted from (Coimbra & Salema, 1994)

Amaranth grain is lenticular in shape with the diameter of 1.0-1.5 mm covered by a rigid hull. Embryo is peripheral and surrounds the starchy perisperm of amaranth seed (Fig. 2.11). These morphological properties have great effect on the

processing of pseudocereals. Embryonic tissue contains more than three-fourths of the oil and a starchy perisperm consisting of more than two-thirds of the seed weight (Sun, et al., 1995). According to Coimbra and Salema (1994) lipid bodies are present in both embryo and endosperm cells. Cytochemical analysis revealed that the embryo and endosperm cells had homogenous distribution with protein bodies inserted in lipid matrix. Berganza (2003) reported that in embryo fraction of amaranth seed is about 20% of oil and germ fraction represents around 25% of the weight of the seed. Carbohydrates are stored as starch in the plastids of the perisperm cell and there is no starch in the embryo or in the endosperm cells. Carbohydrates also are present in the cell wall as polysaccharides. Amaranth seeds contain a unique starch with granules having smooth surface and approximate size of 1 micron.

Amaranth oil and lipophilic constituents

Lipids are very important nutritive constituents of *Amaranthus* seeds, with triacylglycerols (TAGs), phospholipids, squalene, and lipid-soluble vitamins such as tocopherols being the main components in the lipophilic fraction. Various minor components, such as phytosterols, waxes, and terpene alcohols have also been reported in different *Amaranthus* species. The content of all these components in amaranth seeds primarily depends on plant species and cultivar, whereas the extractable amount of lipids also depends on their isolation procedure and the applied solvent. It has been reported that TAGs in the lipid fractions of *A. caudatus* and *A. cruentus* seeds isolated with petroleum ether constituted 80.3% and 82.3%. Phospholipids represented 10.2% and 9.1%, diacylglycerols 6.5% and 5.1%, monoacylglycerols 3.0% and 3.5%, and the content of squalene was 4.8% and 4.9% of the oil, respectively (Gamel, et al., 2007). The lipophilic fraction may be extracted from amaranth seeds with nonpolar organic solvents, such as hexane and petroleum ether using standard procedures, or with dense gases, mainly sub- or supercritical carbon dioxide (SC-CO_2). The oil may also be isolated from amaranth seeds by pressing; however, in this case, the recoveries are remarkably lower. Some latest studies reported the content of lipids in amaranth seeds from 12 accessions was from 10.6% to 16.7% on the dry basis of the seeds. The yield of the oil in Soxhlet extraction may vary from 6.35 to 8.9% according to particle size (Lyon & Becker, 1987), (Barba de la Rosa, et al., 2009).

Písaříková and others (2005), determined that the content of fat in selected varieties of *A. cruentus, A. hypochondriacus, A. caudatus* and *A. hybridus* raw seeds were from 73.0 to 81.1 g/kg, whereas after popping, it slightly decreased and was 67.1 to 77.1 g/kg. Bressani and others (1987) observed a 3.9% reduction in the lipid content in *A. caudatus* seeds due to dry heating; however, possible reasons of such findings were not determined. Popping and cooking reduced the lipid contents in *A. caudatus* and *A. cruentus* seeds by 5.6% and 7.7%, and by 1.7% and 3.7%, respectively (Gamel, et al., 2007).

Fatty acids

The composition of fatty acids in edible oils determines their nutritional, technological, and stability properties. Fatty acid composition of amaranth TAGs

was determined in a number of studies. The main fatty acids in all studied amaranth oils were palmitic, oleic, and linoleic. However, their percentage variations in different species and cultivars were quite remarkable. The major fatty acids in the oil from 11 genotypes of 4 grain amaranth species were palmitic (19.1% to 23.4%), oleic (18.7% to 38.9%), and linoleic (36.7% to 55.9%) with the degree of unsaturation (S/U ratio) in the range of 0.26 to 0.32 (He, et al., 2002). The effect of *A. cruentus* variety (5 varieties) and location (3 locations) on the fatty acid content was also studied and it was determined that the percentage of palmitic acid was in the range of 17.06 to 21.35, stearic 3.05 to 3.80, oleic 20.26 to 32.01, and linoleic 33.56% to 43.88%. However, statistical evaluation of average contents did not reveal any significant differences neither between the varieties nor between the localities (Berganza, et al., 2003). Fatty acid profiles of amaranth TAGs isolated from 21 *Amaranthus* accessions (8 species) were compared to those of barley, corn, buckwheat, oat, lupin, and wheat and it was concluded that amaranth oil was most similar to buckwheat and corn oils (Budin, et al., 1996). Therefore, the content of fatty acids may change during processing. For instance, it was reported that during puffing or popping of *A. cruentus* seeds, the percentage of unsaturation decreased from 75.5% to 62.3%, the quantity of linoleic acid decreased from 46.8% to 27.0%; however, the percentage of squalene increased by 15.5% (Singhal & Kulkarni, 1990).

PLL, POL, OLL, OOL, POO, and LLL were identified as the main TAGs in 3 amaranth species by Jahaniaval and others (2000).

Leafy parts of amaranth, as of many other vegetables, contain low amounts of lipids and therefore were rarely analyzed for their composition. It was reported that in the lipophilic fraction of *A. retroflexus* vegetables isolated with hexane from the crude aqueous alcoholic extract yielding 15%, linoleic and linolenic acids constituted $4.19 \pm 0.35\%$ and $3.71 \pm 0.29\%$ (Conforti, et al., 2012).

Squalene

Squalene is hydrocarbon and an intermediate triterpene (Fig. 2.12) in the cholesterol biosynthesis pathway; its biological and pharmacological activities as well as potential uses in cosmetic dermatology were comprehensively reviewed by Huang, et al. (2009). Shark liver oil is considered as the richest squalene source; however, reasonable amounts are also found in olive, wheat-germ, palm, amaranth seed, and rice bran oils. Squalene, as the main component of skin surface polyunsaturated lipids, shows some advantages for the skin as an emollient, antioxidant, hydrating, and antitumor agent. It is also used as a material in topically applied formulations such as lipid emulsions and nanostructured lipid carriers (Huang, et al., 2009).

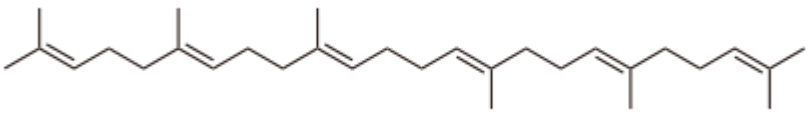


Figure 2.12. Chemical structure of squalene ((6E,10E,14E, 18E) 2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22 hexaene)

Amaranth is a rich source of squalene; therefore, many studies were focused on the isolation and determination of this component in various plant species and cultivars. The content of squalene in *A. cruentus* of 5 varieties and 3 locations was in the range of 2.26% to 5.67% (Berganza, et al., 2003); its concentrations in total lipids of 11 genotypes of 4 grain amaranth species ranged from 3.6% in *A. hypochondriacus* to 6.1% in *A. tricolor* (He, et al., 2002). Conforti, et al., (2005) demonstrated that the squalene content in the seeds of "Oscar blanco" and "Victor red" varieties of *A. caudatus* varied 2.2% and 7.5%, respectively. SCE-CO_2 was also used for the isolation of squalene and it was reported that the effects of solvent temperature and pressure on squalene yield were different. The highest squalene yield (0.31 g/100 g grain) and concentration in the SC-CO_2 extract 15.3% was obtained at 20 MPa and at 50 °C, although the oil yield under these conditions was only 2.07 g/100 g. (He, et al., 2003). The yield of squalene from *A. paniculatus* grains isolated by SCE-CO_2 was dependent on particle diameter, extraction pressure, temperature, and time; maximal yield of 1.36 mg/g was achieved at the highest 55 MPa pressure and 100 °C during 90 min of particles diameter 0.75 mm, solubility of squalene was also estimated by Chrastil equation (Bhattacharjee, et al., 2012). Using RSM squalene-enriched extracts contained up to 12.3% of squalene (0.71 to 1.81 g/100 g seeds). In the same study, it was also observed that at 40 °C, 35.8 MPa, and CO_2 and 20 °C flow rate of 2.9 SL/min in 30 min squalene concentration was the highest (14.43%) and oil yielded up to 6.5 g of oil/100 g, whereas continuing the same extraction the total amount of isolated squalene did not change, while oil content increased up to 15.5 g/100 g after 110 min, thus resulting in a decrease of squalene concentration in the final extract by its dilution with TAGs. The squalene-enriched fraction may also be obtained by gradual decrease of solvent pressure in the extraction system separators. These findings conclude that squalene solubility in SC-CO_2 is better than that of oil and squalene-enriched fraction may be obtained by including multistage extraction the extraction. Using this operation would be possible to obtain purified fractions of squalene in first step and the rest compounds in next step.

Processing of amaranth seeds may have some influence on the distribution and content of squalene; Cooking caused a slight increase in the squalene content of *A. caudatus* and *A. cruentus*, while popping increased the squalene content by 26.5% and 14.5%, respectively. The processing stability of squalene in amaranth and the antioxidant capacity of the oil-rich fraction of amaranth were studied and it was reported that squalene was stable during all of the continuous puffing up to 290 °C and roasting up to 150 °C for 20 min (Tikekar, et al., 2008).

Tocopherols and tocotrienols

Vitamin E is a group of lipid-soluble compounds possessing antioxidant activity (Fig. 2.13). The most active form of vitamin E is RRR alfa tocopherol which possesses the strongest activity. Vitamin E is present in all oil-containing seeds; however, their content and composition are quite different in different plant species and varieties. It was observed that the content of tocols was different in the seeds of different origin. Comparing with many cereal grains, amaranths accumulate significant amounts of both β-and γ -tocotrienols, although α-, δ-tocopherols in

lower amounts. Later, vitamin E profiles of 21 amaranth accessions belonging to 7 species were compared to those of barley, buckwheat, corn, lupin, oat, and wheat oils (Budin, et al., 1996). In this study, the contents of α, β, δ-tocopherols, α, γ , δ-tocotrienols, and the sum of γ-tocopherol and β-tocotrienol were in the ranges of 0.78 to 2.95, 0.71 to 6.74, 0.11 to 2.05, 0.00 to 0.11, 0.00 to 0.06, 0.00 to 0.03, and 0.06 to 0.68 mg/100 g seed (wet basis), respectively.

The differences in the content of tocopherols between the same species from different locations were shown for seed oil of wild *A. caudatus* from Ecuador and *A. caudatus* of Italian origin: SCE-CO2 at 40 MPa was the most efficient extraction method in terms of tocopherol yield, and the seeds of Ecuadorian genotype contained higher levels of tocopherols than Italian samples (Bruni, et al., 2001).

Traditional methods were compared with SCE-CO_2 and the use of ultrasound as a coadjuvant in the extraction process; qualitatively acceptable results in the extraction of tocols were obtained in the case of using ultrasound equipment more rapidly and more economically (Bruni, et al., 2002). The highest yield of tocopherols (317.3 mg/kg seeds) was obtained at 55 MPa by adding 5% of cosolvent ethanol; however, fractionation of extracts by pressure decrease in the separators was not very effective; the highest concentration of tocopherols in the richest fraction was 7.62 mg/g, which was only by 23% higher than in the nonfractionated extract.

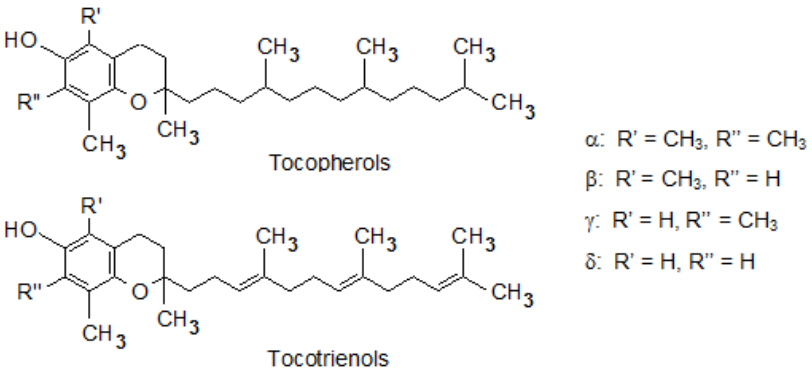


Figure 2.13. Chemical structure of tocopherols and tocotrienols

Sterols and other lipophilic constituents

Sterols are commonly present in oils and fats; however, the data on sterol composition in *Amaranthus* spp. are rather scarce. Fifteen sterols were quantified in *A. cruentus* from Austria; cholesterol, brassicasterol, campestanol, stigmastadienol, sistostanol, and stigmastadienol being in trace levels, whereas the others methylen-cholesterol, campesterol, stigmasterol, campesterol, clerosterol, β-sitosterol, avenasterol, stigmastenol, and avenasterol constituted 0.3, 1.6, 0.9, 24.8, 42.0, 1.3, 2.0, 15.2, and 11.9%, respectively (León-Camacho, et al., 2001). A number of saturated (13) and unsaturated (10) with 1 double bond hydrocarbons (C21-C33)

were reported in *A. cruentus* crude oil by high-resolution GC-MS at the concentrations ranging from 1.91 to 64.99 ppm in the same study.

The total content of polycyclic aromatic hydrocarbons (PAHs) in cold pressed amaranth oil was reported 101.60 ± 3.22 mg/kg, with dominating light PAHs (phenanthrene, anthracene, fluoranthene, and pyrene) which constituted 81.55 ± 1.93 mg/kg (Ciecierska & Obiedziński, 2013).

Others important components of amaranth

Proteins and amino acids

Amaranth seeds are a good source of high-quality proteins. Easily digestible albumins and globulins are the main components of highly nutritive amaranth seed proteins. According to Bressani, (1989) Seeds of *A. caudatus* contain an average 16% protein, with an ideal amino acid balance. Flour made from such seeds can complement the protein intake. Chemical composition and nutritional properties of amaranth, quinoa, and buckwheat proteins, as well as their evaluation from the allergenicity point of view, were reviewed by comparing them with other protein sources (Schoenlechner, et al., 2008). Rajyalakshmi and Geervani (1994) determined protein content in *A. paniculatus* seeds of 22 g/100 g. According to Zheleznov et al. (1997) a 13% to 21% variation of protein was observed in wild and cultivated forms of amaranth seeds.

Proteins of amaranth leafy parts were not studied so intensively, although their nutritional value was shown to be also quite high. Protein contents were evaluated in the foliage of 61 accessions of grain and vegetable types *Amaranthus* (10 species in total): variation for leaf protein was 14 to 30 and 15 to 43 g/kg (fresh weight) in vegetable and grain types, respectively. Leaf protein had a well-balanced amino acid composition with high content of Lys (40 to 56 g/kg) (Prakash & Pal, 1991).

Amaranth proteins contain high amounts of some essential amino acids; however, their contents depend on plant species and cultivar. The content of Lys in proteins of 14 selections of *A. caudatus, A. hybridus, A. cruentus,* and *A. hypochondriacus* varied from 0.73% to 0.84%, with Trp values ranging from 0.18% to 0.28% (Bressani, et al., 1987). An essential amino acid index of amaranth protein (90.4%), which is almost comparable with egg protein decreased to 85.4% after heat treatment by popping at 170 to 190 °C for 30 s; the significant decrease of the essential amino acid (Val and Leu) contents was also observed.

Starch and others carbohydrates

Polysaccharides constitute the main compositional part of amaranth seeds, starch being the main component in this fraction. In general, amaranth seeds contain 65% to 75% starch, 4% to 5% dietary fibres, 2 to 3 times higher content of sucrose in comparison to wheat grain, and nonstarch polysaccharide components (Burisová, et al., 2001). The contents of low-molecular-weight carbohydrates in *A. cruentus* and *A. caudatus* were reported in the following ranges (g/100 g): sucrose (0.58 to 0.75), glucose (0.34 to 0.42), fructose (0.12 to 0.17), maltose (0.24 to 0.28), raffinose (0.39 to 0.48), stachyose (0.15 to 0.13), and inositol (0.02 to 0.04) (Gamel, et al., 2006).

Dietary fibres

Usually dietary fibre is presented as total (TDF), insoluble (IDF), and soluble (SDF). Amaranth seeds are a good source of dietary fibre, which primarily depends on plant species and variety. Another study showed that dietary fibre content in the "Centenario" variety of *A. caudatus* was higher (16.4%) than in the "Oscar Blanco" variety (13.8%) (Repo-Carrasco-Valencia, et al., 2009). Amaranth contained more than 25% water-insoluble β-(1,3)-D-glucan (lichenan), which was less than in oats but higher than in the other 41 analyzed samples of cereals and pseudocereals (Hozová, et al., 2007). The content of resistant starch in amaranth was determined to be 0.65% (González, et al., 2007), and it increased after extrusion-cooking and fluidized heating (González, et al., 2007), whereas during cooking and popping, it decreased (Gamel, et al., 2005).

Polyphenols

Several studies were focused on polyphenols in various *Amaranthus* species, which resulted in the identification of several phenolic acids, flavonoids, and their glycosides. Rutin is the main quercetin glycoside present in amaranth (Fig. 2.14). The total amount of phenolic acids in *A. caudatus* grains was 16.8 to 59.7 mg/100 g, the proportion of soluble phenolic acids was 7% to 61% (Repo-Carrasco-Valencia, et al., 2010). Free phenolic acids were isolated from *A. caudatus* and *A. paniculatus* with ethanol; significant differences in their profiles of both species were observed (Klimczak, et al., 2002). Rutin content in 5 amaranth species ranged from 0.08 (seeds) to 24.5 g/kg dw (leaves), *A. hybrid* and *A. cruentus* being the best sources of rutin (Kalinova & Dadakova, 2009). Steffensen et al. (2011) studied the content of flavonoids: rutin, isoquercitrin, and nicotiflorin, and also the phenolic acids: protocatechuic, vanillic, 4-hydroxybenzoic, *p*-coumaric, syringic, caffeic, ferulic, and salicylic, and the variations among genotype, species, and location of amaranth. Gallic acid was the main phenolic acid found in the seeds and sprouts of *A. cruentus* var. "Rawa" and var. "Aztek": *p*-hydroxybenzoic, *p*-coumaric, vanillic, caffeic, and cinnamic acids were found in the seeds, whereas *p*-coumaric, ferulic, and syringic acids were present in the sprouts.

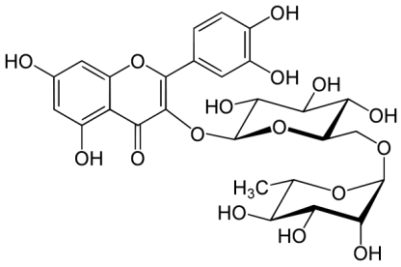


Figure 2.14. Chemical structure of glycoside rutin

The sprouts contained rutin as the main flavonoid and smaller amounts of isovitexin, vitexin, and morin, whereas orientin, morin, vitexin, isovitexin, and traces of neohesperidin and hesperidin were detected in the seeds (Paśko, et al., 2008). Detailed studies of the content of quercetin and rutin in different amaranth anatomical parts (leaves, stems, flowers, and seeds) of 12 plant accessions belonging

to 5 *Amaranthus* species, and their variations during plant vegetation, demonstrated that leafy parts of the plants contained many times higher amounts of flavonoids than the seeds. There were also remarkable variations between the species and even between the varieties belonging to the same species: for instance, the content of rutin was as high as 30.65 g/kg dw in *A. retroflexus* leaves before harvest, whereas *A. tricolor* contained remarkably a lower amount of rutin, from 0.459 (flowers) to 2.62 g/kg (leaves before harvest) (Kalinova & Dadakova, 2009).

Others microconstituents

Raw amaranth seeds are almost favourless. The main volatile compounds of raw seeds isolated with a dynamic headspace procedure were 2,4-dimethyl-1-heptene, 4-methylheptane. Total concentrations of quantified volatile constituents were between 2.2 and 68.9 μg/g of dried sample (Gamel & Linssen, 2008). The contents of total folate in amaranth ranged from 52.8 to 73.0 μg/100 g dw , its content in the bran fractions was more than 2 times higher than in the flour fractions (124% compared with 57%) (Schoenlechner, et al., 2010). Carotenoids in the foliage of 61 accessions of the grain and vegetable types of *Amaranthus* (10 species in total) varied fr3om 90 to 200 mg/kg in vegetable types and from 60 to 200 mg/kg in the leaves of grain types (Prakash & Pal, 1991). Among the green leafy vegetables, *Amaranthus* species are a rich store house of vitamins, including carotene, vitamin B6, vitamin C, riboflavin, folate, as well as essential amino acids and dietary minerals like Ca, P, Fe, Mg, K, Cu, Zn, and Mn (Musa, et al., 2011). The contents of P, Na, K, Mg, Ca, Cu, Fe, Mn, and Zn were similar among 14 selections of *A. caudatus, A. hybridus, A. cruentus* and *A. hypochondriacus* (Bressani, et al., 1987).

2.5. Deduction

Without respect to a number of publications performed in analysis of amaranth, scientific studies on the optimisation of green process from amaranth is a rather scare. To the best of our knowledge RSM was not applied for the optimisation of SCE-CO_2 parameters in order to obtain the highest oil yield and to test the established optimal parameters for the determination of oil yield, fatty acid composition and squalene content in various amaranth accessions.

The studies on the enrichment and fractionation of lipophilic fraction of amaranth seeds by SCE-CO_2 are fragment and not exhaustive. Application of cosolvent and separators operating at different conditions were not tested for this purpose until now. The antioxidant properties of SCE-CO_2 extracts of amaranth were not evaluated previously. Therefore it is important to characterised antioxidant and phytochemical properties of different plant genotypes cultivated in Lithuania applying new analysis, extraction and fractionation techniques.

Literature survey also shows that the composition of tocols and the content of squalene in amaranth may vary in a wide range and SCE-CO_2 may be considered as a promising technique for the fractionation of lipophilic fraction of amaranth in order to obtain the products enriched with bioactive compounds. However, there is a lack of systematic studies in the selection of process parameters and schemes, for instance involving separators and polar cosolvents.

III. MATERIALS AND METHODS

3.1. Accelerated solvent extraction of lipids from *Amaranthus* spp. seeds and characterization of their composition

Materials

Yellow colour seeds of *Amaranthus* named 'Peckaus geltonukai' used for extraction with organic solvents, were obtained from the A. Peckus farm in Lithuania. In addition, the grains from 10 plant cultivars: 'Raudonukai', 'Geltonukai', 'Rudžiai', 'Margiai', 'Rožiniai', 'B-3', 'B-4', 'B-6', 'B-11' and 'B-15', which were grown in the experimental fields of Lithuanian Research Centre of Agriculture and Forestry, Institute of Agriculture were kindly donated by Prof. A. Svirskis. One sample was purchased from the local shop of healthy foods and was labeled as 'Bio'; it is distributed by Infinity Foods Co-operative Ltd (UK), country of origin Bolivia. All solvents used for extractions were of analytical grade and those used for analysis were of chromatography grade.

Milling and particles characterization

The seeds of amaranth were ground in a laboratory mill (Miag, Braunschweig, Germany), at smallest gap (≈0.7 mm). Milling gap was selected from previous published studies (Sun, et al., 1995) in order to crush only bran and avoiding crushing perisperm. During the milling process the temperature of the milled amaranth didn't exceed 30 °C. After grinding, the flour was separated using two different hole size sieves 230 μm and 500 μm. Coarse particles of perisperm were manually separated from the finer bran particles and husk, obtaining three particles fractions (≤230, 230-500 and ≥500 μm). Size distribution of amaranth particles was measured on a particle size analyzer (Mastersizer, Hydro 2000S (A), United Kingdom) operating on a laser diffraction method, which is widely accepted as a standard technique. Particle assessment was performed by the following guidance of ISO13320-1 (1999). Water was used as a dispersant for wet analysis, dispersant refractive index was 1.33, and particle refractive index was 1.53. Physicochemical properties as water, protein (N×5.85) (Salcedo-Chávez, et al., 2002), fat and ash content of the three amaranth seed particle fractions, were determined according to AOAC methods (1995).

Solvent extraction

Soxhlet extractor (Behr Labor-Technik, Düsseldorf, Germany) was used as a standard technique according to the AOAC reference method (1995) using hexane as a solvent. Twenty grams of ground seeds 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 solvent was removed in a rotary vacuum evaporator at 40 °C and the residue was weighed by analytical balances.

Pressurized solvent extraction (PLE) was performed on a Dionex ASE 350 system (Dionex, Sunnyvale, CA, USA). The samples consisting of 10 g milled seeds were mixed with diatomaceous earth (1:4) and placed in a 34 ml Dionex stainless-

steel cell (2.9 cm diameter). Hexane was used as a solvent to extract the oil. The cells were equipped with a stainless steel frit and a cellulose filter at the ends of the cell to avoid solid particles clogging in the collection vial. The following conditions were used for the extraction: cells were preheated 5-6 min to ensure that the samples reached thermal equilibrium at 10 MPa pressure before static extraction with 3 cycles of extraction. Extraction time (t) and temperature (T) were selected as independent variables in order to optimise the yield of amaranth oil.

Preheating time was included in total extraction time. A flush volume of 100% of the cell was used and finally the cell was purged for 60 seconds with nitrogen to collect the extract in the collection vial. The system pressure was held constant at 10.0 MPa during all the extractions. The solvent was removed in a rotary vacuum evaporator at 40 °C and the residue was weighed by analytical balances.

Extractions were replicated three times.

Fatty acid profile and squalene content

Fatty acid composition of seed oil obtained by PLE at optimal conditions was analyzed by gas chromatography (GC). Fatty acid methyl esters (FAME) were prepared by using BF_3 catalyst according to the official AOAC method with slight modifications (1995). FAMEs were analyzed on a HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) equipped with a flame ionization detector and 100 m length with i.d. 0.25 mm, 0.20 μm film thickness fused silica capillary column SP^{TM}–2560 (Supelco, Bellefonte, PA, USA). Analysis parameters were as follows: injection temperature 220 °C; detector's temperature 240 °C; split ratio 100:1; oven temperature was programmed 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 isothermal for 5 min; carrier gas, helium 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.

Squalene as a triterpene hydrocarbon belongs to the unsaponifiable fraction of amaranth lipids; therefore it remains unchanged in the FAME sample and is well separated from the other peaks in FAME chromatogram. Squalene as well as others FA content was expressed in GC area percent.

HPLC analysis of amaranth lipid fraction

A high performance liquid chromatography (HPLC) was used for the qualitative determination of squalene and triacylglycerol (TAG) composition of seed oil according to the official EEC method (1991) with slight modifications. HPLC system (Perkin Elmer Series 200, Shelton, CT, USA) equipped with vacuum degasser series 200, micropump series 200, column oven series 200, UV/Vis detector series 200. Samples were injected using manual injector. Analysis was used under the following conditions: RP-C18 column Altima, particles size 5μm, length 150 mm, internal diameter 2.1 mm (Alltech, Deerfield, IL, USA); 215 nm wavelength; gradient elution with mobile phase A - water, B - ACN / methyl-tert-butylether (9:1), 0-5 min 87% (B), 5-30 min 100% (B), post time 5 min 87% (B); injection volume, 5 μl; flow rate 0.8 mL/min. Oil samples were prepared by

dissolving 100 mg/2 mL (THF). Squalene was identified by comparing the retention time of peak to that of pure squalene.

LC-MS method was used for the identification of individual TAGs. LC-MS system consisted of Waters 1525 binary pump (Waters, Milford, MA, USA), equipped with a manual 7725i Rheodyne injector (Rheodyne Rohnert Park, CA, USA), Waters 996 PDA detector, and Waters ZQ2000 mass spectrometer (Waters, Milford, MA, USA). The separation of TAGs was performed on the same column, as described in squalene analysis method; however the eluents were adapted to MS conditions. A mixture of 18% 2-propanol in methanol was used with added modifiers 0.1% acetic acid, 0.05% ammonium acetate and 0.001% sodium acetate, as proposed by Zeb and Murkovic (2010). Column was heated to 60 °C and the eluent flow rate was set to 0.5 ml/min. MS was operating in ESI positive mode, other parameters were as follows capillary voltage 4 kV, cone voltage 150 V, cone gas flow 120 l/h, desolvation gas flow 350 l/h, desolvation temperature 350 °C, source temperature 120 °C.

Experimental design

The RSM using small central composite design (CCD) was employed to determine the influence of two independent variables on the yield and to identify optimum conditions for seed oil extraction (Hossain, Barre-Ryan & Martin-Diana, 2011). Face centered design using software Design-Expert trial version 8.0.6.1 (Stat-Ease Inc., Minneapolis, MN, USA) was used for data analysis and quadratic model building. Two independent variables were extraction time and extraction temperature with three levels chosen for each variable (Table 3.1).

Table 3.1. Levels of independent variables

Analytical factors	Coded symbols	Factors levels		
		-1	0	+1
Extraction temperature (°C)	T	50	70	90
Extraction time (min)	t	10	15	20

The complete design consisted of 13 experimental runs. Extractions at every experimental point were performed in triplicate and in random order.

3.2. Supercritical carbon dioxide extraction of amaranth seeds by response surface methodology and characterization of extracts

Materials

The same seeds samples of *Amaranthus* spp. named ‘Peckaus geltonukai’ and other species of amaranth as in chapter 3.1 were used for the extraction with supercritical carbon dioxide. The grain was ground using the same methodology as mentioned in chapter 3.1.

Carbon dioxide (99.9%) was obtained from Gaschema (Jonava r., Lithuania). Squalene (99% purity) was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). FAME mixture as a standard obtained from Supelco™ 37 (Supelco, Bellefonte, PA). The solvents used for extractions were of analytical grade and those

used for analysis were of chromatography grade. Helium was obtained from Elme Messer Lit (Vilnius, Lithuania).

Supercritical CO_2 extraction (SCE-CO_2)

Taking into account the measured characteristics of the particles obtained in our study as well as literature data, the fraction ≤230 μm, containing the highest amount of oil, was selected as the most suitable for SFE optimisation. The SCE-CO_2 experiments were carried out using supercritical fluid extractor Helix (Applied Separation, USA). Each extraction was performed using 20 g of amaranth flour sample, which was filled in the vessel between two layers of defatted cotton wool, in both ends, to avoid the particles clogging in the system. The samples were loaded into 50 ml thick-walled stainless cylindrical extractor vessel with an inner diameter of 14 mm and a length of 320 mm. The temperature of the extraction vessel was controlled by a surrounding heating jacket. The flow rate of CO_2 in the system was controlled manually by the micro-metering valve (back-pressure regulator). The volume of CO_2 consumed was measured by a ball float rotameter and a digital mass flow meter in standard liters per minute (SL/min) at standard state (P_{CO2}=100 kPa, T_{CO2}=20 °C, ρ_{CO2}=0.0018 g/ml). The extracts were collected in glass bottles. The conditions for extraction were set as follows: extraction time 60-120 min, pressure 30-40 MPa, extraction temperature 40-60 °C, flow rate of CO_2 2-3 SL/min. A static time of 10 min was maintained constant in all the extraction runs. Static extraction time was included in total extraction time.

Experimental design

Response surface methodology (RSM) using central composite design (CCD) was employed to determine the effect of four variables on the extract yield and to identify optimum conditions for seed oil extraction (Li, et al., 2010). For data analysis and model establishing the software Design-Expert trial version 8.0.6.1 (Stat-Ease Inc., Minneapolis, MN) was used. Extraction pressure (P), temperature (T), time (t) and CO_2 flow rate (F) were chosen as independent variables with five levels for each of them (Table 3.2) (Firatligil-Durmus & Evranuz, 2010). The number of experiments is defined by the formulae:

$$N = (2^f+2f+c) \quad (3.1)$$

where f is the number of factors, c is the number of center points.

Table 3.2. Levels of independent variables

Analytical factors	Coded symbols	Factors levels [a]				
		-2	-1	0	+1	+2
Extraction temperature (°C)	T	30	40	50	60	70
Extraction time (min)	t	30	60	90	120	150
Extraction pressure (MPa)	P	25	30	35	40	45
CO_2 flow rate (SL/min)	F	1.5	2.0	2.5	3.0	3.5

[a] Levels -2, 0, +2 are values given from the software Design-Expert. Levels -1 and +1 are chosen factors values.

The complete design consisted of 30 experimental runs with sixteen factorial points, eight axial points and six center points (1). The data obtained from the CCD design was fitted with a second order polynomial model, which can be expressed as:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{4} \beta_{ij} X_i X_j \qquad (3.2)$$

where Y is the predicted response, β_0 is a constant, β_i, β_{ii}, β_{ij} are the coefficients for linearity, X_i and X_j are independent variables.

Statistical significance of the model and model variables was determined at the 5% probability level (p<0.05). The adequacy of the model was determined by evaluating the lack of fit coefficient and the Fisher test value (F-value) obtained from the analysis of variance.

Extractions at every experimental point were performed in triplicate and in random order.

3.3. Supercritical carbon dioxide extraction of squalene and tocopherols from amaranth and assessment of extracts antioxidant activity

Materials

Black colour seeds of *Amaranthus* called 'Peckaus raudonukai' were kindly donated by Mr. A. Peckus's farm in Lithuania.

Carbon dioxide and nitrogen (both 99.9%) were obtained from Gaschema (Jonava region, Lithuania), modifying solvent ethanol (food grade) was from company Stumbras (Kaunas, Lithuania), HPLC grade solvents for chromatographic analyses were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Reference compounds, squalene (99%) and tocopherols, DL-α-T (99.9%), rac-β-T (90%), γ-T (99%) and δ-T (95.5%) were purchased from Supelco Analytical (Bellefonte, PA, USA). Standard stock solutions of tocopherols and squalene were prepared in the HPLC mobile phase. Ascorbic acid (ACS grade) was purchased from Across Organics (Geel, Belgium), potassium hydroxide (KOH; ACS grade) from Lachema (Neratovice, Czech Republic), 95% (v/v) n-hexane (ACS grade) from Reachem Slovakia (Bratislava, Slovakia) and ethyl acetate from Stanlab (Lublin, Poland). Fluorescein (FL) sodium salt was obtained from Fluka Analytical (Sigma-Aldrich), 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), [2,2-azobis(2-methylpropionamidine) dihydro-chloride] (AAPH) were from Sigma-Aldrich Chemie. Randomly methylated β-cyclodextrin (RMCD) (Trappsol, pharmacy grade) was purchased from CTD Holdings, Inc. (High Springs, FL, USA).

Sample preparation and extraction by SC-CO_2

The seeds were ground using the same methodology as mentioned in chapter 3.1.2. Unlike in the previous experiments, ground seeds were not sieved.

The extraction was performed in a supercritical fluid extractor Helix (Fig. 3.1) using 500 ml volume extraction vessel (Applied Separation, Allentown, PA). Each extraction was performed using 0.12 kg of amaranth flour, which was placed in the

70 mm diameter and 95 mm length plastic basket with porous stainless steel filter at the bottom, to avoid particles clogging. The temperature was controlled by band heaters around vessel body and monitored by the thermocouple inside the extraction vessel. The flow rate of CO_2 in the system was controlled manually by the micro-metering valve (back-pressure valve). The extracts were collected in 60 ml glass bottles attached to the discharge tube of the micro-metering valve. The volume of consumed CO_2 was measured by a ball float rotameter and a digital mass flow meter in standard litters per minute (SL/min) at standard parameters: P_{CO2}=100 kPa, T_{CO2}=20 °C, ρ_{CO2}=0.0018 g/ml.

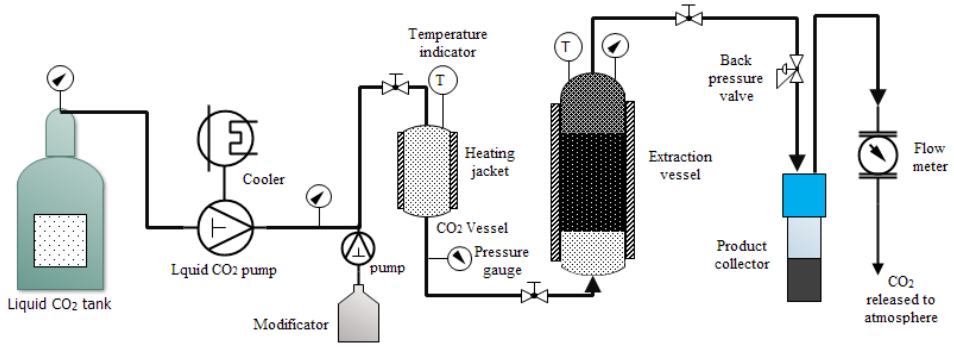


Figure 3.1. Scheme of SC-CO_2 extractor Helix with 500 ml extraction vessel without separators

The experiments were performed in two series: first, extraction without separators in order to select the pressure for obtaining the highest content of tocopherols and squalene by using a cosolvent; second, extraction and fractionation with separators for obtaining the fractions enriched with target compounds. During the first series of experiments extraction time (180 min), temperature (40 °C) and CO_2 flow rate (0.32 kg/h) were kept constant, whereas extraction pressures were 15, 25, 35, 45, 55 and 65 MPa. The cosolvent was pumped using liquid pump, Series 1500 (Applied Separation, Allentown, PA) and mixed with CO_2 before extraction vessel by adding 2 and 5% of cosolvent ethanol. Considering that the CO_2 flow rate is 0.32 kg/h, cosolvent flow is 0.14 and 0.36 ml/min as 2 and 5% respectively. The extracts after extraction with a cosolvent were flushed with nitrogen for 60 min to remove the residues of ethanol.

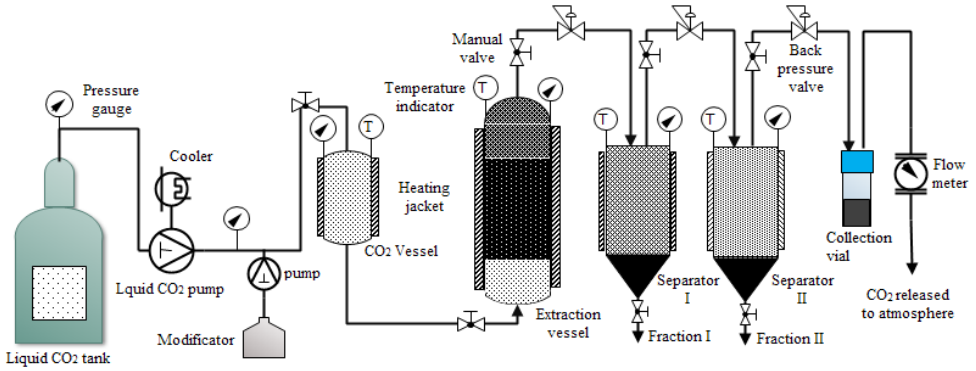


Figure 3.2. Scheme of SC-CO_2 extractor Helix with 500 ml extraction vessel with separators

To obtain enriched with tocopherols fractions, the experiments were carried out using two separators coupled in a sequence to the extractor (Fig. 3.2). The fluid and the dissolved compounds were transported through the separators, where the solvatation power of the fluid decreased by decreasing the pressure. Three fractions were collected. The temperature was constant in both separators (40 °C), while the pressure varied from 45 MPa to atmospheric. The last fraction was collected in the vial at ambient conditions. A static time of 10 min, which was included in total extraction time, was maintained constant in all runs. The lipophilic extracts obtained after extraction were immediately analysed.

Pilot scale extraction was performed in order to scale-up process from 0.5 L volume extraction vessel to 10 L volume extraction vessel. Five kg of ground amaranth sample was loaded into the 145 mm diameter and 490 mm length stainless steel basket with porous stainless steel filter at the bottom. Extraction conditions 40 °C, 35 MPa, CO_2 flow rate 13.5 kg/h and time 180 min. Two fractions were collected gradually decreasing pressure at similar conditions as in AS, in first separator temperature was 40 °C and pressure in the range of 35-20 MPa and 35-10 MPa in second separator pressure was maintained 20-5 MPa and 10-5 MPa respectively.

Pilot-scale (PS) equipment (Applied Separation, Allentown, PA, USA) equipped with 10 L extraction vessel and two separators in series surrounded by heating belt in order to maintain appropriate temperature during extraction (Fig. 3.3). The solvent used for the extraction is recycled for the economical reasons. Extraction pressure and temperature were used the same for the AS and PS although solvent flow rate and extraction time were selected calculating the S/F ratio as scale-up criterion and kept constant 8.1 kg CO2/ kg for 180 min in both extraction process, where S is solvent mass required for the extraction (kg) and F is feed mass (kg). Another parameter U/F ratio as a function of time was also kept constant 2.7 h in both process, where U is the solvent flow rate (kg/h). Bed height to diameter ratio (H/d) of PS is 3.24 and AS 1.35. Solvent residence time (t_{RES}) in the PS vessel is 1 min and in AS vessel 1.81 min. Superficial solvent velocity (u) of the PS is 2.3×10^{-3} m/s and AS is 2.61×10^{-4} m/s.

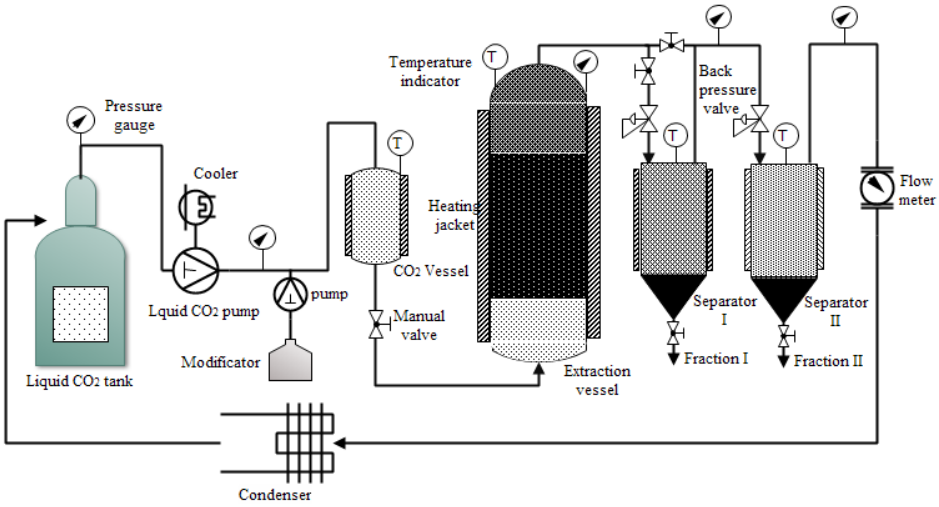


Figure 3.3. Scheme of SC-CO_2 pilot scale 10 L extractor

Isolation of unsaponifiables

The crude oil sample contains some carbohydrates, proteins and minerals that interfere in HPLC separation. Saponification and heating are used to modify sample matrices and by this to increase the accessibility of all unsaponifiables by the solvent (Xu, 2000). Extracted oil samples were subjected to hot saponification according to Ryynänen et al. (2004) with slight modifications. The extract (0.1 g) and antioxidant ascorbic acid (0.05 g) were placed in a screw-capped tube with 5 ml of ethanol (95%) and 0.5 ml of potassium hydroxide (80%) and vortexed 60 s. The test tubes were flushed with nitrogen 60 s, capped and placed in a 70 °C water bath for 30 min periodically mixing. After reaction the tubes were cooled in an ice bath 5 min., 3 ml of deionized water and 5 ml of hexane:ethyl acetate mixture (99:1) were added and again vortexed 60 s. The suspension was then extracted four times with 5 ml of hexane:ethyl acetate mixture. The organic layer was collected and evaporated to dryness with nitrogen; the residue was dissolved in 2 ml of HPLC mobile phase. The samples prepared in such a way were analysed immediately. Saponification was performed in triplicate for each sample.

Determination of tocopherols and squalene by high performance liquid chromatography (HPLC)

An HPLC was used for the quantitative determination of squalene and tocopherols in the saponified sample according to Gruszka et al. (2007) with slight modification. Perkin Elmer Series 200 HPLC system was equipped with C30 reverse-phase column (particle size 5 µm, length 250 mm, i.d. 4.6 mm) applying isocratic elution with acetonitrile:methanol:dichlormethane (72/22/6, v/v/v). Injection volume was 20 µl and flow rate 1 ml/min. Tocopherols were detected using fluorescence detector at 290 nm excitation and 330 nm emission; the analytes eluted in 40 min: α-T at 13.5 min, β-T at 11.5 min, γ-T at 11.0 min and δ-T at 9.5 min. Squalene was detected by UV detector at 214 nm wavelength, its elution time

was 17 min. For tocopherols saponified samples were diluted to a final concentration of 1.6%, while for squalene they were diluted to 0.24%. Squalene and tocopherols were identified by comparing the retention time of peaks to those of pure standard solutions, which were prepared at different concentrations using mobile phase; for tocopherols 0-10 μg/ml and for squalene 0-1 mg/ml. HPLC conditions were set the same as mentioned before. The calibration curves (peak area versus injected amount) were used to determine the quantity of tocopherols and squalene in the samples. Analyses were performed in triplicate.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay has been adapted to measure lipophilic and hydrophilic antioxidants using 7% RMCD solution (50% acetone/50% water, v/v) to solubilise the antioxidants. The L-ORAC method with slight modifications was used to study the antioxidant capacity of amaranth extracts (Huang, et al., 2002). Ten mg of oil were dissolved in 1 ml of 7% RMCD solution to a final concentration of 0.0625%. The 7% RMCD solution was used as a blank. The samples (25 μl) and fluorescein (120 μl, 14 μM) solutions were placed in the 96 wells black opaque microplates with transparent flat-bottom. The microplates were sealed and incubated for 15 min at 37 ºC, afterwards the AAPH solution as a peroxyl radical generator (26 μl; 240 mM) was added manually with a multichannel pipette. The microplate was immediately placed in the FLUOstar Omega fluorescent reader (BMG Labtech GmbH, Offenburg, Germany). Microplate with the samples was shaken prior to each reading. Fluorescence measurements at excitation wavelength 485 nm and emission wavelength 510 nm were recorded every 66 s, in total 120 cycles. At least three independent measurements were performed for each sample. Raw data were analysed using software Mars (BMG Labtech GmbH, Offenburg, Germany). Fluorescein and AAPH solutions were prepared fresh daily. Antioxidant curves (fluorescence versus time) were normalized and the area under the fluorescence decay curve (AUC) was calculated as

$$AUC = 1 + \sum_{i=1}^{i=120} \frac{f_i}{f_0}, \qquad (3.3)$$

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

The final ORAC values were calculated by using a regression equation between the trolox concentration and the net area under the curve (AUC). A series of solutions of trolox, used as a reference antioxidant, in the concentration range of 0-200 μM were prepared for the lipophilic assay. Pure squalene as a reference compound was dissolved in 7% RMCD solution to a final concentration of 0.02%. The antioxidant activity was expressed as trolox equivalent (TE) antioxidant capacity in μmol TE/g oil and g seeds.

Oxipres method

Lipophilic extracts isolated in two separators by PS extractor were further analysed by accelerated oil stability test Oxipres (ML Oxipres, Mikrolab Aarhus

A/S). The Oxipres apparatus was used to study oxidative stability of lipophilic extract according to Trojakova et al. (1999). Oil stability was determined adjusting its induction period, h (IP). Oil oxidation was analysed at 120 °C, adding 5 g of sample and filling system with oxygen (O_2) at 0.5 MPa. IP was calculated according to oxidation kinetic curve.

Statistical analysis

Mean values and standard deviations were calculated from at least three replications using MS Excel 2007. Analysis of variance (one-way ANOVA) was used to test the differences among group means, followed by the 'Duncans' *post hoc* test to compare the means that showed significant variation ($p<0.05$). Analyses were performed using STATISTICA 8.0 software (2007).

3.4. Antioxidant properties and preliminary evaluation of phytochemical composition of different anatomical parts of amaranth

Materials

Amaranthus plant material was collected in July 2011 in Dotnuva region (Lithuania) in a flowering state; the plants were kindly donated by Prof. A. Svirskis (2003). The leaves, flowers and stems were separated and dried at ambient conditions in the dark. The seeds were obtained from Peckus farm (Alytus region, Lithuania). Dried samples were kept in the dark before further handling.

The solvents used for extraction were of analytical grade. Rutin hydrate (95 %), 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), microcrystalline cellulose (20 μm), fluorescein sodium salt, [2,2-azobis(2-methylpropionamidine) dihydro-chloride] AAPH, Folin–Ciocalteau's reagent solution, gallic acid and HPLC grade solvents used for chromatographic analysis were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

Sample preparation and extraction

Dried leaves, flowers and stems were ground by ultra centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.2 mm hole size sieve. The seeds were powdered in a laboratory mill (Miag, Braunschweig, Germany). All ground samples were additionally sieved by using 0.23 mm sieve. ASE was performed in a Dionex ASE 350 system (Dionex, Sunnyvale, CA) from 20 g of powdered material, which was mixed with diatomaceous earth (4:1) and placed in a 66 ml Dionex stainless-steel cells, equipped with a stainless steel frit and a cellulose filter at the both ends to avoid solid particles in the collection vial. Extractions were performed at 70 °C temperature and 10.3 MPa pressure in 25 min; the cell was rinsed with 100 % cell volume of extraction solvent, afterwards the solvent was purged out from the cell with N_2 during 120 s.

The extraction was performed sequentially using solvents of increasing polarity, hexane, acetone and a mixture of methanol/water (70:30 v/v) in order to fractionate

antioxidant compounds present in the dried materials according to their polarity. Hexane was used to remove lipophilic substances, which were not used in further analysis. The following extracts were obtained: FA, FM, LA, LM, SA, SM, SDA, SDM. Organic solvents were removed in a rotary vacuum evaporator at 40 °C while water was evaporated in the freeze dryer.

The extracts of plant samples after evaporation of solvents were additionally kept under nitrogen flow for 20 min. Samples were collected in dark glass bottles at -18 °C until analysis.

Measurements of antioxidant activity

$ABTS^{\bullet+}$ cation radical assay

TEAC assay was used to determine RSC of amaranth extracts as described previously Re et al. (1999) with slight modifications. The assay is based on decolourisation of the $ABTS^{\bullet+}$ radical possessing characteristic absorption maximum at 734 nm in the presence of antioxidants. The working solution of $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate; two stock solutions were mixed in equal quantities and kept for 14-16 h. The working solution was further diluted with a mixture of ethanol:water (50:50 v/v) to obtain the absorbance of 0.70±0.02 at 734 nm.

Plant extracts or trolox solutions (3 µl) were reacted with 300 µl of the $ABTS^{\bullet+}$ solution during 30 min and the absorbance was read at 734 nm in a FLUOstar Omega reader (BMG Labtech, Offenburg, Germany). A series of trolox solutions (150-1500 µM) were used for calibration. The percentage RSC of $ABTS^{\bullet+}$ was calculated by the formulae:

$$[(Abs_{control}-Abs_{sample})/(Abs_{control})]\times 100 \qquad (3.4)$$

where $Abs_{control}$ and Abs_{sample} are the absorbances of $ABTS^{\bullet+}$ in control mixture with methanol and the extract, respectively.

The TEAC values were calculated from the calibration curve and the RSC values were expressed in µmol trolox equivalents (TE) per g dry weight (DW) plant material and extract (µmol TE/g).

$DPPH^{\bullet}$ radical scavenging assay

The method is based on scavenging of free $DPPH^{\bullet}$ radical by the antioxidant, which results in a decrease in absorbance at 515 nm (Brand-Williams, et al., 1995). Briefly, 3 µl of extracts or trolox solutions were mixed in the microplate wells with 300 µl of $DPPH^{\bullet}$ solution. The measurements were performed after 30 min at 515 nm wavelength using a FLUOstar Omega reader. Plant extracts were diluted with methanol to the final concentration of 1-3%. A series of trolox solutions (50-1000 µM) were used for calibration. The final results were expressed as µmol trolox equivalents per g DW plant material and extract (µmol TE/g).

Oxygen radical absorbance capacity assay (ORAC)

The advantage of ORAC assay is that it uses a biologically relevant radical source. The peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product which can be quantified. The reaction was carried out in 75 mM phosphate buffer (pH 7.4); a stock solution of fluorescein was prepared according to Prior et al. (2003), the samples were prepared by dissolving plant extracts in methanol. Prepared samples or trolox (25 µl) and fluorescein (120 µl; 14 µM) solutions were placed in the 96 wells black opaque microplates with transparent flat-bottom. The microplates were sealed and incubated for 15 min at 37 ºC. After incubation AAPH solution as a peroxyl radical generator (25 µl; 240 mM), was added manually with a multichannel pipette. The microplate was immediately placed in the FLUOstar Omega fluorescent reader. The plate with the samples was shaken prior to each reading. Fluorescence measurements (excitation wavelength 485 nm; emission wavelength 510 nm) were read every 66 sec, in total 90 cycles. Raw data were analyzed using software Mars (BMG Labtech GmbH, Offenburg, Germany). Fluorescein and AAPH solutions were prepared fresh daily. Aqueous solutions of trolox were used for calibration (12-200 µM). Antioxidant curves (fluorescence versus time) were normalized and the area under the fluorescence decay curve (AUC) was calculated using formulae 3.3. The final ORAC values were calculated by using a regression equation between the trolox concentration and the net area under the curve (AUC). The antioxidant activity was expressed in µmol trolox equivalent antioxidant capacity per g DW plant material and extract (µmol TE/g).

Measurement of total phenols content (TPC)

The TPC was determined in the extracts using the method of Singleton and Rossi (1965) with slight modifications. Ten µl of appropriate dilutions of the extracts or gallic acid solutions were oxidized with 190 µl Folin-Ciocalteau's reagent solution in deionised water (1:13). The reagents were mixed, allowed to stand for 3 min at room temperature and then neutralized with 100 µl of 7 % sodium carbonate. The mixture was vortexed for 90 min and the absorbance was measured at 765 nm in the FLUOstar Omega reader. The TPC was calculated using gallic acid calibration curve and expressed in mg gallic acid equivalents per g DW plant material and extract (mg GAE/g DW).

Assessment of antioxidant capacity by QUENCHER assay

The measurements of the total antioxidant capacity using modified $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and TPC methods were applied directly to the solid particles of amaranth as described by Pastoriza et al. (2011). All assays were carried in the same way as described for the extracts isolated with solvents. In $ABTS^{\bullet+}$ scavenging assay 10 mg of the powdered sample were weighed in a testing tube and diluted with 40 µl of methanol. The reaction was started by adding 5 ml of $ABTS^{\bullet+}$ reagent. The mixture was vortexed for 2 min to facilitate the surface reaction, centrifuged at 10500 *g* for 3 min, and 300 µl of optically clear supernatant was transferred to the microplate. The $DPPH^{\bullet}$ scavenging assay was performed similarly to the $ABTS^{\bullet+}$ assay with some modification as described previously. In ORAC assay 10 mg of the

powdered sample was transferred to a test tube and the reaction was started by adding 5 ml of fluorescein. The mixture was kept at 37 °C for 15 min and then 175 µl of prepared solution was transferred to the microplate and 25 µl of AAPH solution added.

For TPC measurement 10 mg of the sample were transferred to test tube filled with 2.9 ml Folin-Ciocalteau's reagent solution. The reagents was mixed and allowed to stand for 3 min at room temperature. Then the mixture was neutralized by 2.1 ml of 7% sodium carbonate, vortexed for 77 min and centrifuged at 10500 *g* for 3 min; the absorbance was measured at 765 nm.

In all methods, when the samples exerted too high antioxidant activity, they were diluted with microcrystalline cellulose as an inert material. The samples of cellulose-reagent mixture were prepared as control in all measurements using microcrystalline cellulose. Trolox solutions were used to prepare the calibration curve, using microcrystalline cellulose as well. The results are expressed in µmol equivalents of Trolox per g of DW (µmol TE/g DW).

On line high performance liquid chromatography radical scavenging assay (HPLC-DPPH$^{\bullet}$)

An on-line HPLC-DPPH$^{\bullet}$ radical scavenging was used for the detection of active compounds present in amaranth extracts. Initial conditions with some modifications were according to Paśko et al. (2008). LC system consisted of Waters 1525 binary pump (Waters, Milford, MA), equipped with a manual 7725i Rheodyne injector (Rheodyne, Rohnert Park, CA) and Waters 996 PDA detector. The separation of extracts was performed on a Supelco Discovery® HS C-18, particle size 5 µm, length 250 mm and internal diameter 4.6 mm (Supelco Inc. Bellefonte, PA) using gradient elution consisting of A - 2.5% acetic acid and B - acetonitrile. The composition of gradient was set as follow: 0 min 10% B; 0-5 min, from 10 to 20% of B; 5-20 min, from 20 to 25% of B; 20-30 min, from 25 to 45% of B; 30-40 min, from 45 to 10% of B, using the flow rate of 1 ml/min. Twenty µl of the sample was injected for the analysis. The UV detection range for the tested compounds was 210 - 450 nm. The separated constituents were transferred into a post column reaction coil with circulating DPPH$^{\bullet}$ solution. Two chromatograms were recorded simultaneously: absorbance of effluent before the reaction at 210-450 nm and absorbance at 515 nm after reaction of effluent with DPPH$^{\bullet}$. The DPPH$^{\bullet}$ solution in methanol (6×10^{-5} M) was freshly prepared before analysis and continuously supplied into a reaction coil (length 15 m, internal diameter 1.5×0.3 mm) with Agilent 1100 series pump (Agilent Technologies, USA) at a flow rate of 0.6 ml/min. The signals were acquired at 515 nm wavelength by UV-VIS detector (Model SPD-20A, Shimadzu, Kyoto, Japan). Rutin identification was carried out by comparing the retention time with that of the corresponding peak in chromatograms of standard. The extract samples were prepared by dissolving them in methanol to a final concentration of 1-3%.

UPLC/ESI-QTOF-MS analysis

An Acquity UPLC system (Waters, Milford, MA, USA) combined with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was used. The Acquity UPLC was equipped with a binary solvent delivery system, an autosampler with a 10 μL sample loop, a photodiode array (PDA) detector, a column manager, and a data station running the Compass aquisition and data software. An Acquity BEH C18 column (particle size 1.7μm, length 50 mm and i.d. 2.1 mm) was used for separation of compounds at 25 °C. The mobile phase was initially composed of 95 % eluent A - acetic acid (0.4 % v/v acetic acid solution in ultra pure water) and 5 % B (acetonitrile), followed by a gradient 0-8.5 min, from 5 to 25 % of B; 8.5-10 min, from 25 to 100 % of B; 10-12 min, from 100 % of B. In the PDA detector the effluent was monitored at 254 nm and further was introduced directly into the UHR-TOF mass spectrometer equipped with an ESI source at the flow rate of 0.3 mL/min. Analysis were achieved using the Compass 1.3 (HyStar 3.2 SR2) software. MS experiments were performed in negative ionization mode. The capillary voltage was maintained at +4000 V with the end plate offset at -500 V. Nitrogen was used as the drying and nebulizing gases at a flow rate of 10.0 L/min and a pressure of 2.0 bar, respectively. Nitrogen was introduced into the collisional cell as the collision gas. The peaks were identified by the characteristic mass spectrometric fragmentation patterns and accurate masses.

Statistical analysis

The results are given as mean values and standard deviations, which were calculated from at least three replicate measurements using MS Excel 2003. Analysis of variance (one-way ANOVA) was used to test any differences in antioxidant activities, followed by the Duncans' post hoc test to compare the means that showed significant variation ($p<0.05$) (STATISTICA 8.0 software, 2007).

3.5. Optimisation of rutin isolation from different anatomical parts of *Amaranthus paniculatus* using accelerated extraction with green solvents

Materials and methods

Amaranthus paniculatus plants were grown in Lithuania (Varėna district, Panara vilage). Plants were sampeled during flowering stage in 2013. Ethanol used for extraction was extra pure (Scharlau, Scharlab, Spain), methanol of LS-MS grade (VWR, Radnor, Pennsylvania, USA) and ultrapure water. Prior to extraction the solvents were pretreated by ultrasound. Solvents used for the HPLC analysis were of gradient grade and acetic acid ≥99% both obtained from Sigma Aldrich. Rutin hydrate ≥94% (HPLC), powder as a standard was obtained from Sigma-Aldrich.

Dry flowers and leaves of amaranth were mixed and ground using miller (Retsch GM 200, Haan, Germany), stems before grinding were separated from leafes and flowers and not used. Seeds were ground by a conical laboratory mill (Miag, Braunshweig, Germany). Before PLE extraction part of ground seeds were defated using supercritical CO_2 extraction.

Presurised liquid extraction (PLE)

PLE was done using Dionex accelerated solvent extractor ASE-200 (Dionex, Sunnyvale, CA, USA). The samples consisting of 2 g ground sample were mixed with washed sea sand (Panreac, Barcelona) (1:1) and placed in a 11 mL Dionex stainless-steel cell. Ethanol and ultrapure water mixture was used as a solvent to extract active compounds from the samples. The Water and ethanol mixture was prepared at certain concentration and pretreated with ultrasound to remove air. The cells were equipped with a stainless steel frits and cellulose filters at the ends of the cell to avoid solid particles in the collection vial. The following conditions were used for the extraction: cells were preheated 9 min before filling with a solvent, 10 MPa pressure in the cells was maintained during all extractions. Extraction time, temperature and solvent ratio were selected according to the experimental design. Preheating time was not calculated to the total extraction time. The extraction cells were washed with 60% of cell volume and finally purged 60 s with nitrogen. Ethanol was removed by rotary vacuum evaporator and water using freeze-drier. Dry samples were kept in the fridge until analysis.

Conventional extraction as a reference extraction was used ultrasonically assisted extraction (Peng, et al., 2010). 2 g of leaves and flowers ground sample was transfered into the flask and mixed with 60 ml of solvents mixture (90:10, v/v) methanol/ethanol and placed into the ultrasonic bath for 50 min at room temperature (25 °C) preventing from the light.

HPLC analysis of rutin content

Quantification was performed by dissolving crude extracts into water and methanol mixture (50:50). Before analysis all samples were filtered through nylon syringe filters (sartorius, pore size 0.45µm) into HPLC glass vials.

LC system for quantification was equipped with degasser (Agilent 1100 Series), pump (Agilent 1100 Series), autosampler (Agilent 1200 Series), DAD (Agilent 1100 Series) and with a column heater (Cecil, CE 4600). Analysis were performed on the ACE 3, C18-AR, 150×4.6 mm column using gradient elution consisting of solvent A–2.5% acetic acid and solvent B–acetonitrile. The composition of gradient was used as follow: 0 min 10% of B; 0–5 min, from 10–20% of B; 5–10 min, from 20–25% of B; 10–20 min, from 25–45% of B; 20-30 min, from 45–100% of B; 30–35 min, from 100% of B; 35–40 min, from 100–10% of B using the flow rate of 0.8 ml/min. Twenty µl of the sample was injected. During analysis column was termostated at 30 °C. Each chromatogram was recorded at 280 nm and 350 nm, whereas rutin quantification was performed at 350 nm. For quantification of rutin different concentration of rutin standard solutions were prepared. Calibration curve was created by calculating rutin peak area at different concentrations. Rutin standard was dissolved in methanol and later diluted to appropriate concentration.

Identification of phenolics by UPLC-MS/MS

Phenolics identification was carried out on an Accela (Thermo Scientific, San Jose, CA, USA) LC system equipped with degasser, pump, autosampler and PDA detector and coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole

analyzer via an electrospray interface. Analytical conditions used for the phenolics analysis were adopted from quantification analysis. The flow rate was decreased up to 0.4 ml/min and total analysis time 70 min. The solvents were used the same as mentioned in previous analysis eluted according to slightly changed gradient: The diode array detector absorption spectra recorded between 200 nm and 500 nm. Each chromatogram was recorded at 280 nm and 350 nm channels. The MS was operated under ESI negative ionization and using Q1MS mode. Mass determination range was adjusted at 150-850 m/z and scan time 0.38 s.

Supercritical antisolvent fractionation (SAF)

The supercritical antisolvent extraction is the process used to recover solid compounds from a liquid mixture (extract). The extract obtained from the PLE was mixed with SC-CO_2 under constant flow in the pressurised vessel (precipitation vessel). Compounds soluble in SC-CO_2 recover in collector whereas not soluble solids precipitate at the bottom of precipitation vessel (Fig. 3.4). For the fractionation of liquid mixture two pumps were used. The extract was delivered by HPLC pump and CO_2 by liquid CO_2 pump. First fraction of compounds was recovered in pressurised stainless steel precipitation vessel and second fraction in collector at atmospheric pressure. Pressure and CO_2/extract flow ratio were determined from CCD, whereas temperature in precipitation vessel and collector kept constant at 40 °C and room temperature respectively. CO_2 flow rate kept constant during all experimental runs, 0.216 kg/h. Solvent from the first fraction collected in precipitation vessel was removed by freeze-drying and from the second under nitrogen flow. Twenty ml of extract was used in all extraction runs.

Rutin quantification was performed as previous, dissolving 0.01 g extract with 1 ml of water and methanol mixture (50:50).

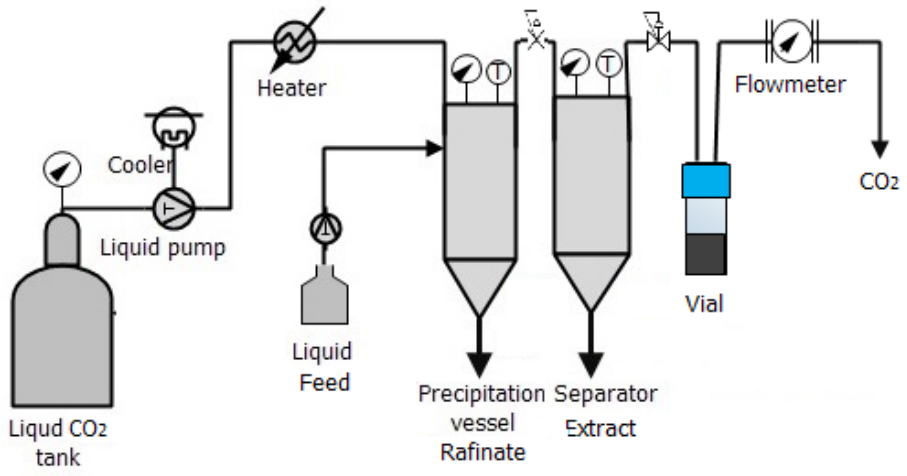


Figure 3.4. Scheme of SAF

Experimental design

The highest rutin content in amaranth leaves and flowers was determined by using response surface design of experiments. The Design-Expert 7.0.0 was used for the experiments evaluation. Rotatable central composite design (CCD) was defined by three independent variables: extraction temperature (T), time (t) and water/ethanol ratio (R) with five levels for each of them. Factors values are presented in table 3.4. The CCD involves 20 experimental runs: 2^3 factorial points, 2×3 axial points and 6 centre points; the order of experiments was randomised. The design runs in a single block. The number of experiments is defined by the formulae 3.1.

Table 3.4. Independent variables of PLE in order to maximize rutin content

Factors	*-1.68*	*-1*	*0*	*+1*	*+1.68*	*Units*
Temperature	50	80	124	168	198	°C
Time	5	8	12.5	17	20	min
Water content	0	20	50	80	100	%

Rotatable CCD was used to obtain the highest concentration of rutin in the rafinate using supercritical antisolvent fractionation (SAF). Two independent variables as antisolvent pressure and feed flow rate with five levels were used to optimise extraction conditions. Factors within five levels are presented in table 3.5. The CCD involves 13 experimental runs: 2^2 factorial points, 2×2 axial points and 5 centre points; the order of experiments was randomised.

Table 3.5. Independent variables values of SAF in order to maximize rutin concentration in the fractions

Factors	*-1.41*	*-1*	*0*	*+1*	*+1.41*	*Units*
Pressure	15	20	25	30	32.1	MPa
Feed flow rate	0.16	0.2	0.3	0.4	0.44	ml/min

IV. RESULTS AND DISCUSION

4.1. Accelerated solvent extraction of lipids from *Amaranthus* spp. seeds and characterization of their composition

Particle sizing and physicochemical properties

Amaranth grains are small, approx. 1 mm diameter spherical particles. Before extraction amaranth grains were ground to powder and separated into 3 fractions using 3 different holes size sieves (230 μm, 500 μm, 1000 μm). Grinding was used to triturate the grain and in this way to increase the specific surface area of the sample and consequently to intensify mass transfer in order of achieving better extraction rate and oil yield. Undisrupted grains were not analyzed because very little yield of extracted oil was previously reported by using organic solvent and supercritical fluid extraction (He, et al., 2003). Particle separation with the standardized sieves is a reference method in the particle size distribution (PSD) analysis. Other existing methods of PSD measurement use the principles of light scattering, acoustic spectroscopy and laser diffraction. Laser diffraction method is one of the most frequently used for sizing small particles. In our study particles sizes were determined using wet analysis method. Distilled water was chosen as a dispersant because particles are not soluble in water, while wetting of the particles by water is quite efficient. Particle size parameters of each fraction are given in Table 4.1, while comparison of the particle distribution in different fractions is shown in Figure 4.1 according to the used sieves.

Table 4.1. Particle size parameters of each milled amaranth seed fraction [a]

Fractions	d (0.1)	d (0.5)	d (0.9)	D [4, 3]	D [3, 2]
Particles ≤ 230 μm	9.5 ± 7.2	77.7 ± 3.1	320.2 ± 3.4	127.0 ± 2.9	17.9 ± 3.7
Particles 230 - 500 μm	4.2 ± 6.7	316.6 ± 4.0	772.7 ± 1.4	336.5 ± 2.3	12.8 ± 3.8
Particles 500 - 1000 μm	3.0 ± 2.2	671.8 ± 5.1	1302.0 ± 2.2	611.5 ± 4.2	11.3 ± 2.2

[a] The values are expressed in terms of percent (%) as mean ± standard deviation of six determinations. D [4, 3] - volume weighted mean, D [3, 2] - surface weighted mean.

It may be observed that in the finest fraction (< 230 μm) the particles of 70–80 and 200–220 μm prevail, in the medium fraction (230–500 μm) the particles of 500–550 μm are dominant, while the largest particle size fraction (500–1000 μm) contain the highest amount of 950–980 μm particles.

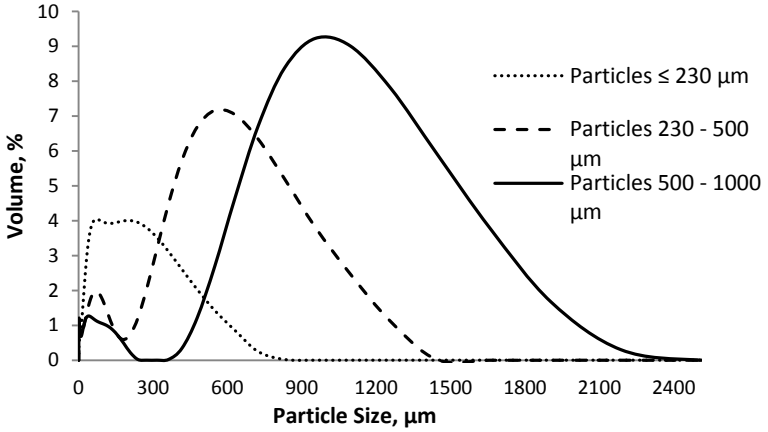


Figure 4.1. Particles size distribution in different fractions

Particle sizing by laser diffraction is influenced by the particle shape; usually the optical model assumes that the particles are spherical (Ma, et al., 2000). According to (ISO, 1999) non-spherical powders can cause bias and errors due to the theoretical assumptions of particle sphericity.

Fat content in the finest particle fraction (≤230 µm) was more than 2 times higher than in the middle fraction and almost 9 times higher than in the coarsest fraction (Table 4.2). The finest particle fraction also had the highest content of crude protein, 25.8%. Sun et al. (1995) analysed oil content of amaranth perisperm and bran milled at various milling gaps using stone mill and determined that milling gap had a significant effect on oil distribution between the bran and perisperm fractions; oil content in the bran fraction varied from 12.3 to 18.4%. Moisture content in the finest particles was 10%, i.e. by 2% lower than in the coarser particles (12%). It is known that the influence of moisture content up to 10% is negligible for SCE-CO_2 (He, et al., 2003). According to Berganza et al. (2003), amaranth seeds consist of about 25% of germ fraction or bran, which contains ≈20% of oil and appropriate milling can efficiently separate the perisperm from the germ. These findings are in agreement with our results. It should be noted that the smallest particles fraction (≤230 µm) also contain germ. In addition, defatted by SCE-CO_2 at optimal parameters amaranth samples from 12 varieties were analysed according to AOAC method 993.13 (1995) by the combustion method using a CE-440 elemental analyzer (Chelmsford, Massachusetts, USA). The samples were accurately weighed into disposable aluminum capsules. Protein content was calculated from the nitrogen content of the material, using a nitrogen conversion factor of 5.85.

Table 4.2. Composition of amaranth seed particles fractions

Samples	Moisture (%)[a]	Protein (%, dw[b])	Ash (%, dw)	Fat (%, dw)
Particles ≤230 µm	10.07 ± 0.03	25.79 ± 0.14	5.29 ± 0.02	14.31 ± 0.51
Particle 230-500 µm	12.10 ± 0.01	11.23 ± 0.43	2.61 ± 0.19	6.43 ± 0.17
Particles ≥500 µm	12.73 ± 0.11	4.44 ± 0.15	1.07 ± 0.01	1.64 ± 0.02

[a] Results are expressed as mean ± standard deviation of triplicate determinations,
[b] dw: dry weight basis.

Solvent extraction of lipids

Solvent extraction is a common method for the measurement of lipid content; therefore Soxhlet method was used as an official reference method for oil extraction from amaranth in our study. The process is very efficient, however hexane elimination after extraction and difficulties in removal of polar and bound lipids from the sample are the major problems. The procedure provides a soaking effect and doesn't permit channeling.

The effect of time and particles size on extraction of oil from the grain was examined (Fig. 4.2). It is evident that oil yield increased remarkably by reducing particles size. Since the smallest particles have bigger surface area and shorter diffusion path, the oil is being isolated much more efficiently comparing to bigger particle size fractions. The oil yield obtained from the finest amaranth fraction (≤ 230 μm) was almost 9 times higher than that from the largest fraction (500–1000 μm) and more than two times higher comparing to the middle fraction (230–500 μm). Generally, the major part of oil was extracted within the first half an hour; afterwards extraction rate reduced and only a small amount of oil was extracted during prolonged process time. It seems that in case of bigger particle size diffusion of oil from the inner particle part to the outer layer which is in contact with solvent becomes negligible.

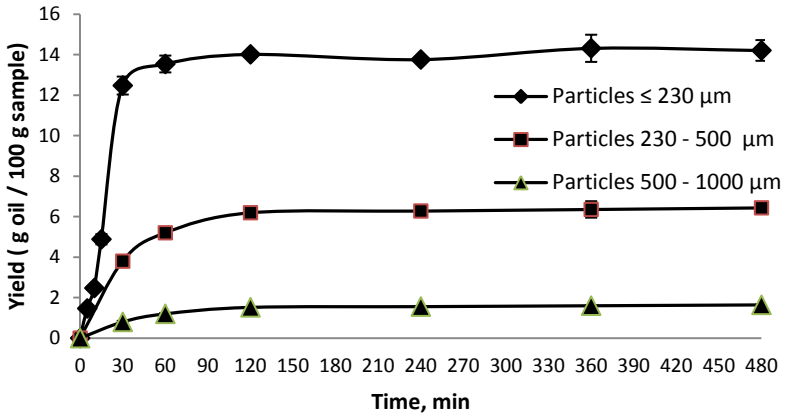


Figure 4.2. Oil yield from different particles size by Soxhlet

Westerman et al. (2006), compared extraction of Amaranthus seed oil by supercritical carbon dioxide with traditional method using organic solvent hexane in a Soxhlet apparatus. The total oil content of milled seed extraction over an 18 h period was 8.1 g oil/100 g sample. He et al. (2002), using traditional extraction method with organic solvents, compared 11 genotypes of four Amaranthus species. The content of obtained oil was from 5.1 to 7.7 g oil/100 g sample. However, they did not indicate particle size and their distribution.

PLE was used to test extraction rates of three particle fractions of amaranth at initial 110 °C temperature (Fig. 4.3). Obtained results were similar to those obtained by Soxhlet extraction; the highest oil content was extracted from the smallest particle size fraction. However, in PLE the extraction rate was remarkably higher;

the main part of extractable lipids was removed from the amaranth during the first 5 min of extraction process.

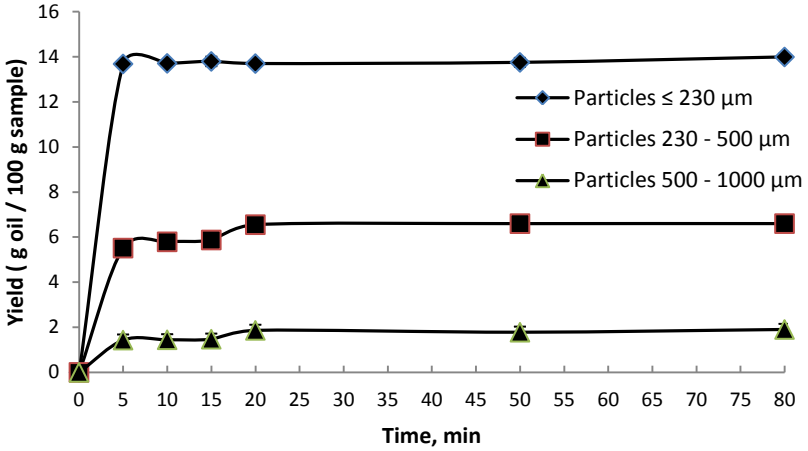


Figure 4.3. Oil yield from different particles size by PLE

Fatty acid composition

Fatty acid composition of seed oil is an important characteristic of nutritive value. Palmitic, stearic, oleic and linoleic acids were major constituents of the analyzed amaranth oils isolated by different methods; however some variations between amaranth accessions may be observed (Table 4.3). Thus, the content of palmitic acid was in the range of 15.69-26.34%, oleic - 20.18-37.12%, linoleic – 37.08-46.96%, stearic – 3.17-4.62%. These fatty acids were reported as the main ones in the all previously studied Amaranthus species. The percentage of other identified fatty acids did not exceed 1%, except for linolenic, which was found in the range of 0.53-1.21%. Arachidic and lignoceric acids occurred in lower amounts; however their content was dependent on the extraction method. Behenic and arachidonic acids were present in low amounts in oil isolated in a Soxhlet or ASE apparatus. Some differences in the content of palmitic and linoleic acids in the oils isolated buy using standard and ASE methods may be observed. For instance, the content of polyunsaturated arachidonic and linolenic fatty was found in the oils extracted in a ASE apparatus. According to He and Corke (2003), there is no typical Amaranthus seed oil; overall average contents of three major fatty acids were 21.3, 28.2 and 46.5% for palmitic, oleic and linoleic acids respectively. This fatty acid profile is similar to oils obtained by ASE method.

Table 4.3. Fatty acid compositions of seeds oil of *Amaranthus* spp. extracted by PLE [a]

Fatty acids		Peckaus geltonukai (*Amaranthus* spp.)		Raudonukai	Bio	Geltonukai	B-11	B-4	B-6	B-3	B-15	Rudžiai	Margiai	Rožiniai
		Soxhlet	ASE (opt.cond.)											
Myristic	C 14:0	0.28	0.26	0.36	0.32	0.18	0.19	0.19	0.16	0.16	0.17	0.13	0.18	0.18
Palmitic	C 16:0	25.9	24.3	26.3	21.7	20.4	15.7	20.6	16.4	14.0	19.5	19.5	18.5	18.5
Palmitoleic	C 16:1	0.42	0.52	0.46	0.33	0.47	0.53	0.32	0.54	0.60	0.42	0.49	0.48	0.48
Heptadecanoic	C 17:0	0.11	0.12	0.12	0.10	0.10	0.08	0.07	0.08	0.08	0.10	0.15	0.09	0.09
cis-10-Heptadecanoic	C 17:1	0.68	0.77	1.25	0.59	0.75	0.84	0.59	0.62	0.67	1.04	0.79	0.86	0.86
Stearic	C 18:0	3.34	3.75	3.20	3.78	4.49	3.24	4.12	3.64	3.17	3.71	4.62	4.00	4.00
Oleic	C 18:1	26.9	27.3	20.2	28.9	32.2	35.6	34.2	36.2	37.2	25.5	31.3	31.6	31.6
Linoleic	C 18:2c	40.2	40.1	45.1	42.0	38.1	41.0	37.1	39.2	41.3	46.9	39.0	40.9	40.9
Linolelaidic	C 18:2t	0.29	0.30	0.25	0.17	0.30	0.34	0.28	0.32	0.31	0.32	0.34	0.33	0.33
Linolenic	C 18:3	0.60	0.51	1.12	0.53	1.00	0.77	0.77	0.81	0.77	0.74	1.21	1.04	1.04
Arachidic	C 20:0	0.53	0.64	0.59	0.80	0.81	0.56	0.77	0.71	0.58	0.81	0.92	0.78	0.78
cis-11-Eicosenoic	C 20:1	0.15	0.20	0.20	0.26	0.65	0.47	0.36	0.39	0.34	0.30	0.34	0.32	0.32
Arachidonic	C 20:4	0.11	0.29	0.10	0.09	0.09	0.10	0.06	0.14	0.12	0.13	0.16	0.14	0.14
Behenic	C 22:0	0.11	0.20	0.21	0.23	0.27	0.19	0.29	0.29	0.23	0.28	0.35	0.30	0.30
Lignoceric	C 24:0	0.23	0.11	0.12	0.12	0.17	0.16	0.20	0.23	0.18	0.19	0.24	0.21	0.21
Saturated		30.5	29.4	30.9	27.1	26.4	20.1	26.3	21.5	18.4	24.8	25.9	24.0	24.0
Monounsaturated		27.46	28.03	20.84	29.5	33.3	36.6	34.7	37.2	38.1	26.2	32.1	32.3	32.3
Polyunsaturated		41.8	41.9	47.8	43.4	40.2	43.1	38.8	41.1	43.2	49.2	41.5	43.3	43.3
Unsaturated/Saturated		2.27	2.38	2.22	2.69	2.79	3.96	2.80	3.64	4.40	3.04	2.84	3.15	3.15

[a] Results shown are mean values from duplicate analyses and are given in %

Lipid fraction analysis by HPLC

Lipid fractions of amaranth isolated by Soxhlet and ASE methods were directly analysed by HPLC. The highest peak in it represents squalene, which was eluting from the column at 11.4 min., while the peaks eluting after 15 min run are attributed to amaranth triacylglycerols (TAGs). It may be observed that this fraction consists of the three main TAGs. Nine TAGs were identified in the oil from amaranth, namely LLL, OLL, PLL, PLO, OLO, POO, OOO, OSO and ALO. Some TAGs were coeluting, and their presence there detected only by the different MS signals. Coeluting compound pairs were PLL with OLL, PLO with OLO, POO with OOO, and OSO (or SOO) with ALO. Their MS spectra corresponded with the ones obtained by Zeb and Murkovic (2010). However we did not detect significant $[M+H]^+$ peaks and only $[M+NH_4]^+$ and $[M+Na]^+$ signals were detected. Spectral data is shown in Table 4.4.

Table 4.4. Identification data of amaranth TAG's by electrospray ionization MS

TAG	$[M+NH_4]^+$	$[M+Na]^+$	Diacylglycerol fragments (m/z)		
LLL	879	902	LL 599	-	-
PLL	873	878	PL 575	LL 599	-
OLL	899	904	LL599	LO 601	-
PLO	875	880	PL575	PO 577	LO 601
OLO	901	906	OL601	OO 603	-
POO	877	882	PO577	OO 603	-
OOO	903	908	OO603	-	-
OSO or SOO	n.d.	910	OO603	SO 605	-
ALO	n. d.	936	LO n.d.	AL n.d.	AO 633

n.d. – not detected.

Squalene content in amaranth seeds

Squalene is one of the most important lipophilic component in amaranth seed. It is usually measured by HPLC, however, in our study its content in addition was measured by GC in FAMEs fractions and expressed in GC area % (Table 4.5).

The content of squalene in the extracted oil and measured by HPLC varied from 4.3 to 6.0 % (w/w), while in FAMEs fractions the percentage of this compound was 3.8-6.9 %. It is not possible to directly compare the quantity of squalene measured by different methods, because GC involves esterification of fatty acids, while HPLC uses direct analysis of the extracted oil. However, it is interesting to note that in some cases the percentage of squalene in FAMEs mixture and measured by GC was equal or very close to the content measured by HPLC. Most likely, the composition of saponifiable and unsaponifiable fractions in the amaranth lipid influences the differences of squalene percentage amount in the total extract and in its FAMEs mixture.

Table 4.5. Squalene content in amaranth measured by HPLC and GC methods[a]

Amaranthus accessions	w/w in oil sample measured by HPLC	GC area % in FAME mixture
Peckaus geltonukai	6.0	5.8
Raudonukai	4.3	3.8
Bio	4.6	3.5
Geltonukai	5.1	5.7
B - 11	5.2	5.2
B - 4	5.2	5.3
B - 6	5.2	5.2
B - 3	5.2	6.7
B - 15	4.8	5.4
Rudžiai	5.3	6.8
Margiai	5.4	5.9
Rožiniai	5.8	6.9

[a] The results are the mean of three determination, SD≤0.3

Optimisation of ASE parameters

The smallest particle fraction was selected for the optimization of ASE parameters to maximize amaranth oil yield. Table 4.6 presents the yields of oil extracted under the thirteen experiments performed in triplicate including 5 central points, while Fig. 4.4 presents response surface plot showing the effect of temperature and time on oil yield.

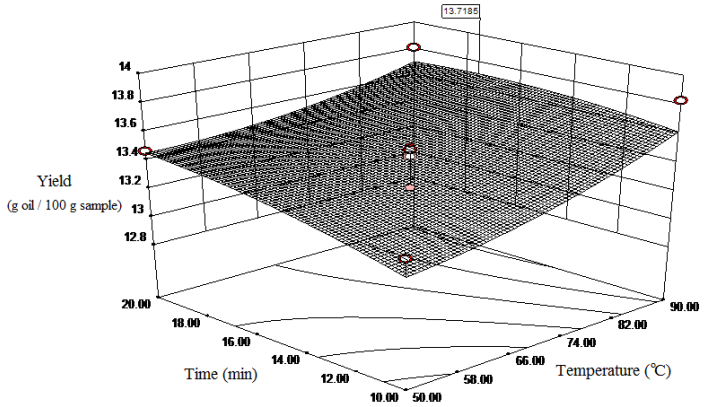


Figure 4.4. Response surface plot

According to the results, the optimal temperature was 90 °C and time 17 min. In this analysis, temperature influence had slightly bigger impact on the extraction rate and oil yield than time. The determination coefficient of model was quite high (R^2 = 0.8170) and the lack of fit value of the model was 0.198, which indicates that the lack of fit was not significant (p>0.05). The model F–value of 6.25 indicates that the model is significant. There is only 1.62 % chance that such model F-value could

occur due to the noise. A significant (p<0.05) effect of temperature was observed on oil yield at quadratic level. The predicted optimal oil yield value (13.72 g/100 g sample) well fitted with the actual experimental value (13.73 g/100 g sample).

Table 4.6. Experimental design and response values

Run	Temperature (°C)	Time (min)	Yield (g oil/100g sample)[a]
1	70	15	13.51 ± 0.21
2	70	15	13.44 ± 0.20
3	110	15	13.98 ± 0.16
4	70	5	12.98 ± 0.09
5	30	15	13.37 ± 0.05
6	50	10	13.28 ± 0.07
7	70	25	13.47 ± 0.26
8	90	20	13.81 ± 0.03
9	70	15	13.45 ± 0.24
10	70	15	13.49 ± 0.08
11	90	10	13.83 ± 0.09
12	50	20	13.47 ± 0.04
13	70	15	13.22 ± 0.14

[a]The yield of oil is the mean ± SD of three experimental runs

Other amaranth samples were extracted using optimized conditions, 17 min extraction at 90 °C. In general, comparing with the previous studies in our work analysed amaranth cultivars contained quite high amounts of lipids, the highest yield, 18.5 g oil/100 g flour was obtained for the commercial amaranth labeled as 'Bio', while the smallest oil content was from the sample 'Raudonukai' (Fig. 4.5). As it was mentioned, in the majority of previously performed measurements of amaranth lipids, particle size was not taken into account; therefore, incomplete extraction could be one of the possible reasons for the lower yields of oil in the previously studied amaranth grains.

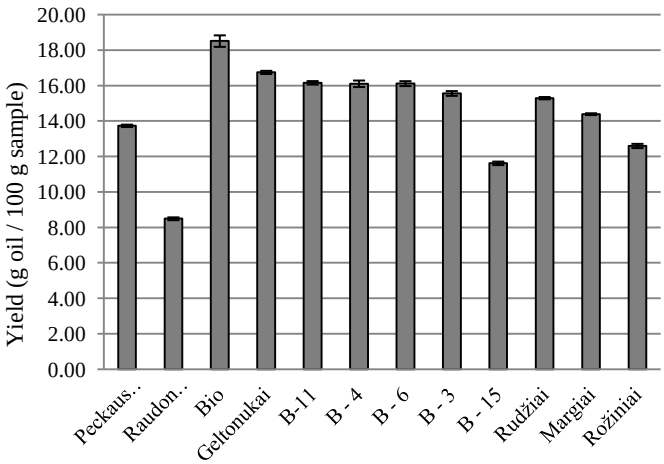


Figure 4.5. Oil yield of amaranth from different verities

4.2. Supercritical carbon dioxide extraction of amaranth seeds by response surface methodology and characterization of extracts

Optimisation of SCE-CO_2 conditions

Central composite design (CCD) was used to optimise four independent variables (pressure, temperature, extraction time, and CO_2 flow rate) at five levels. The oil yield obtained from experiments varied from 7.1 to 15.4 g oil/100 g sample. The optimal conditions for obtaining the highest oil yield were: time 109.9 min, temperature 40.4 °C, pressure 35.8 MPa, CO_2 flow rate 2.9 SL/min. *Amaranthus* spp. oil yield obtained at optimal conditions was 15.43 g/100 g. Validation experiments carried out under optimal conditions gave the value of 15.5 g/100 g, which is not significantly different from the predicted value. The fat content extracted from middle fraction particles (230-500 μm) and the coarsest fraction particles (≥500 μm) under optimal conditions was 6.79 g/100 g grain sample and 2.75 g/100 g grain sample respectively. Oil yield from different fractions was similar to that obtained by solvent extraction.

Response surface plots showing the effect of extraction time, temperature, pressure and CO_2 flow rate on oil yield are presented in Fig. 4.6 (a-f). The graphs were obtained by fixing two variables at optimal parameters, while varying the remaining two variables and predicting the response variable. Fig. 4.6 (a) illustrates the linear effect of extraction temperature and quadratic effect of time on oil yield at a fixed pressure of 35.0 MPa and CO_2 flow rate 2.5 SL/min. Extraction time had the main influence on oil yield comparing with the effect of extraction temperature. Oil yield increased by lowering extraction temperature and increasing time in the selected variables range. Fig. 4.6 (c) shows the effect of temperature and CO_2 flow rate; temperature and flow rate had a linear and minor effect on oil extraction in the selected range at higher temperature, but had higher effect on oil yield at lower temperature. According to Westerman et al. (2006) this was due to the higher density of the solvent and therefore the higher solvent strength at lower temperatures. Increasing temperature also increases the volatility of the solute, while the increase in vapor pressure of the solute reduces solvent power due to the reduced density. Using floured seeds, CO_2 diffusion limitation was minor, therefore increasing CO_2 flow rate increased the yield and extraction rate. Fig. 4.6 (d) presents the influence of extraction pressure and time; it can be observed as a quadratic effect for both pressure and time on oil extraction. Considering all the response it is evident that extraction time and pressure have the highest positive effect on oil extraction, while the effect of CO_2 flow rate and temperature was minor. Previous studies also reported that extraction time and pressure are the most important parameters in SCE-CO_2 of seed oil (Liu, et al., 2009) (Ixtaina, et al., 2010).

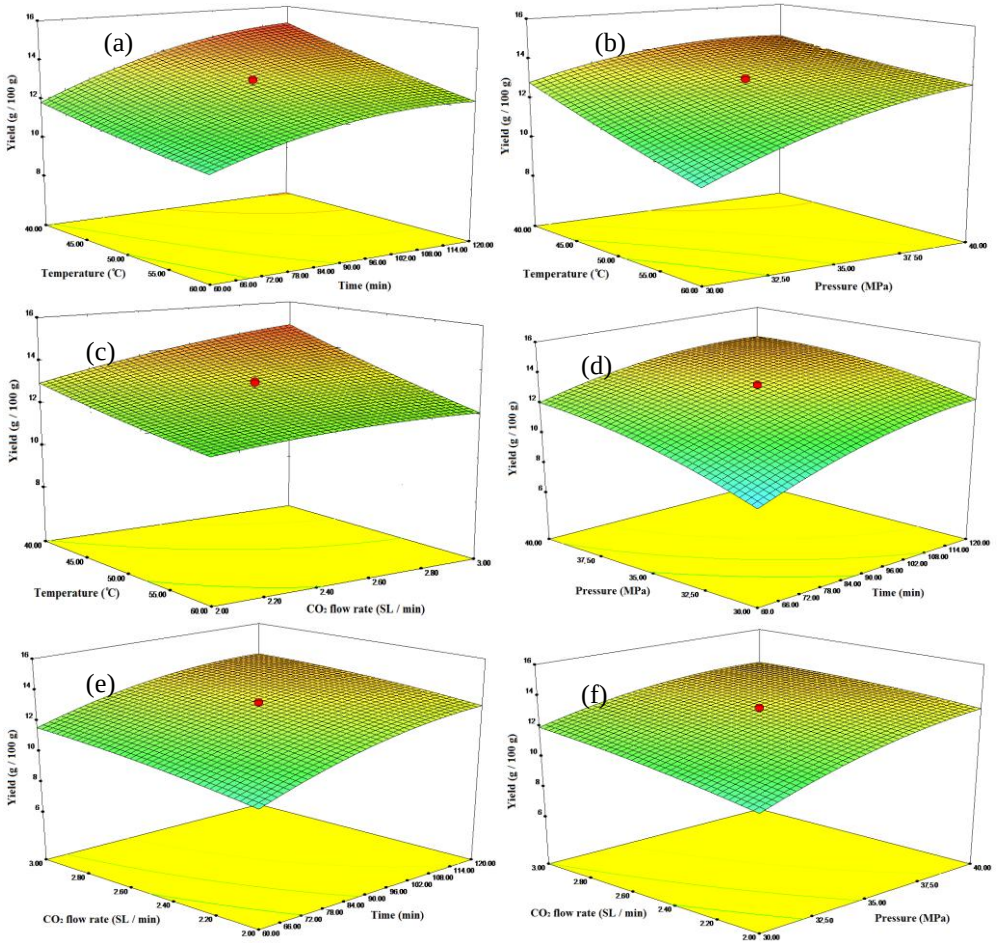


Figure 4.6. Response surface plot

The residue remaining after amaranth extraction by SCE-CO_2 is of interest as sources of other valuable substances, such as high nutritional value proteins. Therefore protein content from amaranth seed varieties was determined from defatted flour using combustion method. Amaranth flour was defatted using SCE-CO_2 at optimal conditions. Daun and DeClercq (1994) compared combustion and Kjeldahl methods for determination of nitrogen in oilseeds. They suggested that nitrogen determination by the combustion method gave more accurate results in oilseeds than the Kjeldahl method. They also found that the combustion method gave higher values than the Kjeldahl method. The crude protein content in 11 amaranth varieties is presented in Fig. 4.7. The highest protein content was obtained from the sample 'Bio' (35.6%) and the lowest one from the sample 'B-15' (26.1%). In comparison protein content in common cereals varies within the range of 13.3-21.5% (Zheleznov, et al., 1997) (Bressani & Garcia-Vela, 1990). Consequently the finest fraction of amaranth seeds contains almost 2 times more protein than not fractionated ground amaranth seed flour.

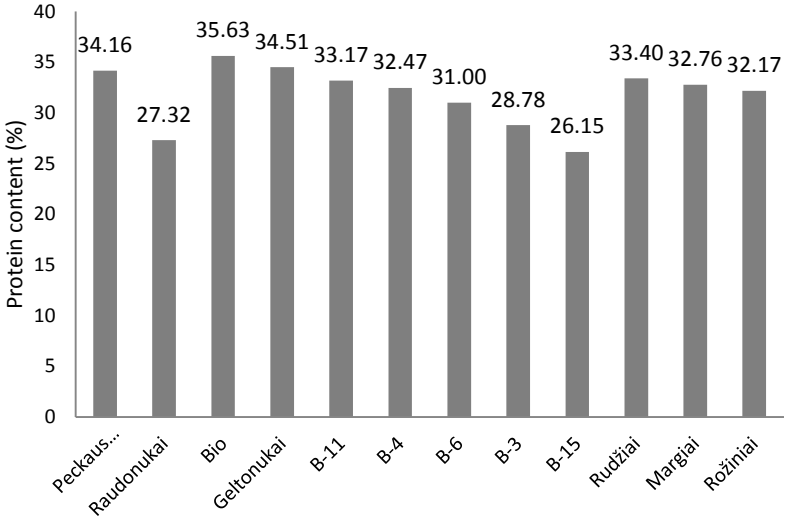


Figure 4.7. The crude protein content of *Amaranthus* spp.

Model analysis

Response surface methodology (RSM) was used to assess the effect of SCE-CO_2 parameters on the total oil yield. Second order polynomial regression model, which is an empirical relationship between dependent variables and the independent test variables (T, t, P, F), is given in the following equation.

$$Y=12.9833-1.0208\times T+1.3125\times t+1.2041\times P+0.5958\times F-0.3062\times T\times t+0.4687\times T\times P-0.3437\times T\times F-0.3937\times t\times P-0.1812\times t\times F-0.3562\times P\times F+0.0989\times T^2-0.6385\times t^2-0.5510\times P^2-0.2010\times F^2 \quad (4.1)$$

Predicted values were calculated using a second order polynomial equation (3.2) and compared with experimental values in Fig. 4.8. The adequacy of the model was evaluated by the total determination coefficient (R^2) value of 0.99, indicating a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2) of 0.98 is in agreement with the predicted coefficient of determination (R^2) of 0.96. The adjusted (R^2) is a measure of the amount of variation about the mean explained by the model.

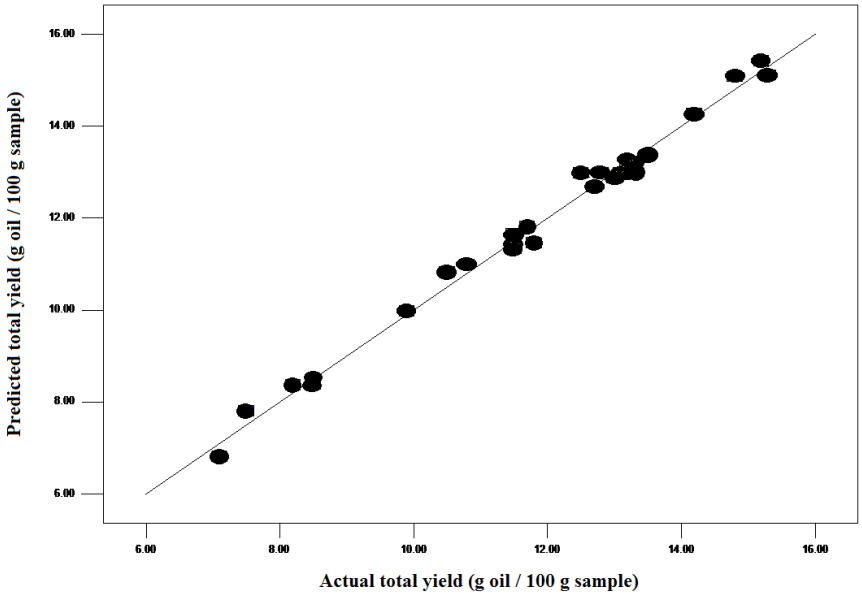


Figure 4.8. Comparison of actual total yield and predicted total yield of oil from amaranth seeds. Predicted values were calculated from equation (4.1)

Model evaluation is presented in the analysis of variance (Table 4.7). The significance of each model was determined using the Student test (*p*-value). The analysis of the quadratic regression models for oil yield showed that the model was significant ($p<0.0001$) with an *F*-value of 108.87 and the "lack of fit" was not significant relative to the pure error, with a *p*-value of 0.6667. The model shows that the factor with the largest effect on oil yield was, extraction time (t) ($p<0.0001$) followed by extraction pressure (P) ($p<0.0001$), extraction temperature (T) ($p<0.0001$) and CO_2 flow rate (F) ($p<0.0001$). Interaction between temperature and pressure (TP) had the highest effect on oil yield and was significant ($p<0.0001$); other interactions between factors also had significant effect on oil yield ($p<0.05$). The second-order terms as time (t^2) and pressure (P^2) was significant ($p<0.0001$).

Table 4.7. Analysis of variance table for response surface quadratic model

Source	Sum of squares	df	Mean square	*F*-Value	*p*-value
Model	140.65	14	10.05	108.87	< 0.0001 significant
T	25.01	1	25.01	271.03	< 0.0001
t	41.34	1	41.34	448.04	< 0.0001
P	34.80	1	34.80	377.13	< 0.0001
F	8.52	1	8.52	92.33	< 0.0001
Tt	1.50	1	1.50	16.26	0.0011
TP	3.52	1	3.52	38.10	< 0.0001
TF	1.89	1	1.89	20.49	0.0004
tP	2.48	1	2.48	26.88	0.0001
tF	0.53	1	0.53	5.70	0.0306
PF	2.03	1	2.03	22.01	0.0003
T^2	0.27	1	0.27	2.91	0.1086
t^2	11.18	1	11.18	121.19	< 0.0001
P^2	8.33	1	8.33	90.26	< 0.0001
F^2	1.11	1	1.11	12.01	0.0035
Residual	1.38	15	0.09		
Lack of Fit	0.84	10	0.08	0.76	0.6667 not significant
Pure Error	0.55	5	0.11		
Corrected Total	142.04	29			

Fatty acid composition of seed oil from different amaranth varieties

The fatty acid (FA) profile of amaranth seeds varieties oil obtained by SCE-CO_2 was analysed by GC (Table 4.8). Unsaturated fatty acids compose up to 75% of the total fatty acids. The dominant FA's in seed oils of amaranth varieties were similar; linoleic (37-50%), followed by oleic (19-34%), palmitic (18-21%) and stearic acids (3-4%). The remaining FA's were found in much lower amounts (0.5–1%). These findings are consistent with the previous reports on the fatty acid composition of seed oil of *Amaranthus* spp. grown in experimental farm of China (2003). In addition, amaranth oil possesses fatty acid profile similar to the oils from cotton seeds and corn (Lyon & Becker, 1987). The FA composition and the ratio of saturated/unsaturated (S/U) FA are important indicators in evaluating nutritional and functional value. The S/U FA ratios from different genotypes were almost similar, on average 0.33 (75% unsaturated fatty acids and 25% saturated fatty acid), excluding the 'Rožiniai' sample with the ratio of 0.37 indicating slightly higher amount of saturated fatty acids. The Highest content of polyunsaturated fatty acids was detected in sample 'Raudonukai' (53.31) and the lowest was in sample 'B-4' (39.19). The highest content of saturated was determined in sample 'Rožiniai' (26.95%) and the highest content of monounsaturated was detected in sample 'B-4' (35.62%).

Table 4.8. Fatty acid compositions of seeds oil of *Amaranthus* spp. extracted by SC-CO_2 [a]

Fatty acids		Peckaus geltonukai	Raudonukai	Bio	Geltonukai	B-11	B-4	B-6	B-3	B-15	Rudžiai	Margiai	Rožiniai
Myristic	C 14:0	0.15	0.18	0.20	0.17	0.13	0.14	0.14	0.14	0.14	0.14	0.15	0.17
Palmitic	C 16:0	19.4	19.7	18.5	19.4	18.5	18.8	18.9	18.6	18.2	19.0	18.5	21.8
Palmitoleic	C 16:1	0.45	0.38	0.32	0.46	0.4	0.48	0.51	0.49	0.49	0.49	0.45	0.55
Heptadecanoic	C 17:0	0.11	0.10	0.11	0.10	0.1	0.10	0.09	0.10	0.11	0.11	0.08	0.13
cis-10-Heptadecanoic	C 17:1	0.76	1.26	0.61	0.76	0.8	0.59	0.59	0.60	1.04	0.77	0.83	1.16
Stearic	C 18:0	3.90	3.44	4.01	4.45	4.6	4.37	4.30	4.50	3.75	4.53	4.32	3.59
Oleic	C 18:1	29.5	21.1	29.8	32.6	33.2	34.7	34.5	33.9	25.7	31.6	30.9	19.4
Linoleic	C 18:2c	41.5	50.5	43.7	38.3	38.5	37.4	37.7	38.5	47.3	39.7	41.1	50.1
Linolelaidic	C 18:2t	0.34	0.30	0.19	0.40	0.36	0.29	0.27	0.32	0.34	0.35	0.37	0.36
Linolenic	C 18:3	0.62	1.15	0.58	1.02	0.77	0.84	0.80	0.75	0.76	1.23	1.11	0.90
Arachidic	C 20:0	0.72	0.82	0.94	0.86	0.97	0.89	0.89	0.93	0.86	0.92	0.87	0.80
cis-11-Eicosenoic	C 20:1	0.28	0.29	0.30	0.68	0.55	0.42	0.36	0.36	0.33	0.35	0.33	0.31
Arachidonic	C 20:4	0.02	0.13	0.15	0.09	0.15	0.02	0.12	0.11	0.10	0.07	0.04	0.07
Behenic	C 22:0	0.02	0.29	0.31	0.29	0.35	0.36	0.36	0.37	0.30	0.34	0.30	0.27
Lignoceric	C 24:0	0.18	0.21	0.18	0.19	0.26	0.27	0.27	0.29	0.21	0.24	0.22	0.18
Saturated		24.5	24.7	24.2	25.51	24.9	24.9	24.9	24.9	23.6	25.3	24.5	26.9
Monounsaturated		30.3	21.8	30.4	33.70	34.2	35.6	35.4	34.7	26.6	32.4	31.8	20.2
Polyunsaturated		43.2	53.3	45.2	40.58	40.6	39.2	39.5	40.1	49.6	42.1	43.5	52.5
Saturated/Unsaturated		0.33	0.33	0.32	0.32	0.33	0.33	0.33	0.33	0.31	0.34	0.33	0.37

Fatty acid compositions of seeds oil of *Amaranthus* spp.

[a] Results shown are mean values from duplicate analyses and are given in %

Squalene content

Amaranth oil contains high concentration of squalene comparing with other commercial oils (Gamel, et al., 2007) (Becker, 1994). Table 4.9 shows squalene content and oil yield in the finest seed particles from different amaranth varieties. Squalene amount in this study varied from 4.6 to 7.6% in oil of amaranth samples, while oil yield varied from 8.5 to 16.7 g/100 g at optimised extraction conditions (40 °C and 35.0 MPa). The differences in squalene and oil yield between amaranth varieties were remarkable. The sample 'Bio' originated from Bolivia distinguished by the highest oil yield (16.7 g/100 g) contained rather low percentage of squalene, 5.65%. The sample of amaranth seeds 'Peckaus geltonukai' contained the highest amount of squalene, 7.6% and average yield of oil 15.5 g/100 g. The smallest amount of oil was determined in sample 'Raudonukai'. He et al. (2002) reported that amaranth seed oil isolated by solvent extraction contains from 3.6% to 6.1% of squalene. He et al. (2003) in further experiments compared the oil yield and squalene concentration of amaranth grain by SCE-CO_2 with those by solvent extraction. They obtained 2.5 times higher squalene concentration (15.3%) in oil, but almost 2.5 times lower oil yield (2.07 g of oil/100 g of grain) using SCE-CO_2 at 50 °C and 200 bar.

Table 4.9. Oil yield and squalene content extracted by SC-CO_2 from amaranth varieties at optimal conditions

Amaranth varieties	Squalene content in oil (%)[a]	Squalene yield (g squalene/100 g sample)	Oil yield (g oil/100 g sample)
Peckaus geltonukai	7.6±0.03	1.17	15.5±0.01
Raudonukai	4.6±0.08	0.39	8.5±0.25
Bio (commercial)	5.7±0.04	0.94	16.7±0.36
Geltonukai	7.1±0.06	1.09	15.3±0.09
B-11	6.8±0.07	1.04	15.2±0.58
B - 4	7.1±0.01	1.09	15.4±0.42
B - 6	6.3±0.03	0.96	15.1±0.30
B - 3	6.4±0.04	0.96	15.1±0.21
B - 15	6.1±0.02	0.65	10.6±0.47
Rudžiai	6.3±0.15	1.81	14.7±0.08
Margiai	6.9±0.03	0.96	13.8±0.18
Rožiniai	6.9±0.05	0.84	12.1±0.22

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

It was also observed that squalene concentration at SCE-CO_2 conditions: 40 °C, 35.8 MPa and CO_2 flow rate of 2.9 SL/min in 30 min, was the highest (14.43%) and oil yielded up to 6.5 g of oil/100 g grain. The total amount of extracted squalene continuing the same extraction didn't change, while oil content increased up to 13.9 g of oil/100 g after 60 min and 15.5 g/100 g after 110 min, thus decreasing squalene concentration in oil. These findings conclude that squalene solubility in SC-CO_2 fluid is better than that of oil. Bhattacharjee et al. (2012) analysed the solubility of squalene in SC-CO_2 under different operating conditions using Chrastil equation. They obtained maximum solubility of squalene by Chrastil equation 160.47 g/kg under operating conditions: 100 °C, 55 MPa, 0.2 L/min in 90 min.

4.3. Supercritical carbon dioxide extraction of squalene and tocopherols from amaranth and assessment of extracts antioxidant activity

Supercritical CO_2 extraction yield and the content of unsaponifiable fraction

The extraction was scaled-up from 50 ml volume extraction vessel to 500 ml volume extraction vessel (Applied Separation, Allentown, PA). Amaranth oil yield obtained using 50 ml extraction vessel at optimal conditions (chapter 4.2) was 4.11 g/100 g seeds. The yield of lipids isolated with hexane in a Soxhlet apparatus was higher (4.7 g/100 g seeds) comparing with SCE-CO_2 obtained oil yield (4.11 and 3.99 g/100 g seeds) in 50 ml and 500 ml extraction vessels respectively (Table 4.10).

Table 4.10. Composition of lipophilic fraction of amaranth seeds isolated by various extraction methods

Methods	Total oil content, g/100 g seeds	Unsaponifiable substances, %	Total squalene content, g/kg seeds	Tocopherols content, mg/kg seeds			
				α-T	β-T	γ-T	δ-T
Soxhlet extraction	4.7±0.02^{c}	21.1±0.63^{b}	2.38±0.12^{ab}	2.14±0.09^{a}	157.2±8.6^{ab}	27.51±1.05^{a}	35.83±1.54^{b}
SCE-CO_2 in 50 ml vessel, 110 min	4.1±0.25^{abc}	12.2±0.53^{a}	3.07±0.29^{b}	6.49±0.32^{c}	170.0±10.5^{ab}	42.46±2.67^{c}	32.14±1.64^{ab}
SCE-CO_2 in 500 ml vessel, 180 min	3.9±0.31^{a}	12.4±0.92^{a}	2.96±0.45^{ab}	5.95±0.30^{c}	177.1±12.6^{b}	40.87±1.96^{c}	33.13±0.96^{ab}

Values represented as a mean ± standard deviation (n=3); different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$).

In general, the yield of amaranth substances increased by increasing the pressure (Table 4.11), however, this dependency was different for the extractions with pure CO_2 and in case of using a cosolvent, which enabled to obtain higher yields almost at all applied pressures. For instance, when 5% of cosolvent was used the yield significantly increased by increasing pressure up to 35 MPa, while further increase did not have significant effect on the extract yield. Thus, the highest yield of 5.12 g/100 g was obtained at 55 MPa and 5% of cosolvent, which was larger than the yield of crude oil, obtained in a Soxhlet apparatus with hexane (Table 4.10).

Table 4.11. Effects of supercritical carbon dioxide pressure and cosolvent on amaranth extract yields and the content of unsaponifiable substances in the extracts.

Pressure, MPa	Extract yield, g/100 g sample	Unsaponifiable substances, %
	pure SC-CO_2	
15	1.37±0.26^{a}	16.35±0.07^{c}
25	3.24±0.51^{d}	11.4±0.42^{a}
35	3.76±0.39^{bc}	12.45±0.21^{ab}
45	3.72±0.22^{b}	13.6±1.27^{b}
55	4.37±0.41^{e}	13.1±0.28^{b}
65	4.00±0.53^{c}	12.75±2.19^{ab}
	2% cosolvent^{z}	
15	2.14±0.08^{a}	13.65±1.06^{b}
25	3.07±0.09^{e}	12.5±0.14^{a}
35	4.62±0.07^{b}	17.5±1.26^{c}
45	4.44±0.12^{f}	19.4±0.64^{d}
55	4.74±0.21^{d}	14.6±1.56^{b}
65	4.71±0.09^{bcd}	11.95±0.92^{a}
	5% cosolvent	
15	3.20±0.09^{a}	12.6±0.89^{a}
25	4.18±0.43^{d}	13.7±1.12^{a}
35	5.07±0.13^{bc}	22.6±1.98^{bcd}
45	5.02±0.53^{bc}	19.4±1.52^{b}
55	5.12±0.24^{c}	25.1±2.15^{d}
65	4.98±0.16^{bc}	14.9±1.27^{a}

^{z} Ethanol was used as cosolvent;
Values represented as a mean ± standard deviation (n=3); different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$).

Saponifiable lipids were removed from the sample before determination of tocopherols and squalene. Tocols may be bound to the other components in the matrix, therefore for their full isolation from the sample they should be liberated during sample preparation. The content of unsaponifiable fraction in the extracts varied with significant difference ($p<0.05$) in a rather wide range, from 11.4 to 25.1% (Table 4.11). The content of unsaponifiable fraction isolated by Soxhlet extraction was significantly higher (21%) than that by SCE-CO_2; however the content of unsaponifiable fraction was higher at higher pressures when a cosolvent was added. The dependence of unsaponifiable fraction in the extract on the amount of cosolvent and pressure seems to be rather complicated. For instance, it may be observed that in case of 2% of modifier the amount of unsaponifiables increased by increasing the pressure up to 45 MPa, whereas in case of 5% cosolvent the amount of unsaponifiables increased by increasing the pressure up to 55 MPa; further increase of pressure resulted in the remarkable reduction of this fraction in the extract. The highest contribution of unsaponifiable fraction was at 55 MPa and 5% of cosolvent (25.1%). Schwartz et al. (1988) using solvent extraction obtained 2.4 times lower amount of unsaponifiable substances from *A. cruentus* comparing with our results by Soxhlet extraction. According to Czaplicki et al. (2011) cold pressed amaranth seed oil contained 7.12% of unsaponifiables matters. It should be noted that the compositional

differences between various amaranth species and varieties may be remarkable and therefore the results obtained in different studies for the same extract characteristics may vary in a large range.

Table 4.12. The concentration of squalene in amaranth oil (g/100 g) extracted by pure SC-CO_2 and its mixtures with ethanol and the yield of squalene obtained from the seeds (g/100 g) at different extraction conditions

Pressure,	pure SC-CO_2		SC-CO_2 + 2% ethanol		SC-CO_2 + 5% ethanol	
MPa	oil	seeds	oil	seeds	oil	seeds
15	14.88 ± 0.31^{d}	0.204	10.99 ± 0.49^{c}	0.235	8.11 ± 0.21^{c}	0.259
25	7.11 ± 0.13^{c}	0.230	6.97 ± 0.26^{b}	0.214	5.73 ± 0.14^{ab}	0.239
35	7.05 ± 0.72^{bc}	0.265	5.85 ± 0.16^{a}	0.270	6.02 ± 0.24^{b}	0.305
45	6.77 ± 0.27^{abc}	0.250	6.18 ± 0.24^{a}	0.274	5.44 ± 0.07^{a}	0.273
55	6.25 ± 0.66^{a}	0.273	5.81 ± 0.15^{a}	0.276	5.65 ± 0.20^{a}	0.289
65	6.28 ± 0.07^{ab}	0.251	5.86 ± 0.11^{a}	0.276	5.62 ± 0.03^{a}	0.280

Results are expressed as a mean ± standard deviation (n=3);
Different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$).

The values of squalene content varied from 5.62 to 14.88 g/100 g oil and from 0.204 to 0.305 g/100 g seeds (Table 4.12). The highest concentration of squalene in oil was determined at 15 MPa and without addition of cosolvent (14.88 g/100 g); however squalene amount extracted from seeds at these conditions was the lowest, 0.204 g/100 g. Although the highest content of squalene from 100 g of amaranth seeds was extracted at 35 MPa and with 5% of cosolvent (0.305 g/100 g) it may be observed that the percentage of this compound in the extracts decreased by adding cosolvent because the total extract yield increased and squalene was diluted by other substances. The differences in squalene content in oil extracted at higher than 35 MPa pressures were not significant ($p<0.05$). These findings may be used for obtaining the fraction with high squalene content in oil, almost 15% at 15 MPa with pure CO_2. Considering low extract yield at these conditions, the rest materials may be extracted at higher pressure with cosolvent for a more exhaustive extraction of other substances. According to Bruni (2001) the differences in the solubility of constituent present in the material could be due to coextracted substances that may interfere with squalene extraction. Bhattacharjee et al. (2012) studied squalene solubility under different conditions of SC-CO_2 and suggested that the amount of squalene increased with increasing pressure and decreasing temperature. The highest yield of squalene (1.36 mg/g seed) in their study was obtained at 55 MPa and 100 °C. Comparing to our results, squalene yield extracted by pure SC-CO_2 was 2.73 mg/g seeds at 55 MPa and 40 °C temperature, while adding 5% ethanol at the same conditions squalene yield increased only by 5% (2.89 mg/g seeds). He et al. (2003) reported that squalene content in the extracts was from 0.05 to 0.31 g/100 g, while the highest yield was obtained at 20 MPa and 50 °C; increasing pressure up to 30 MPa and the temperature up to 70 °C the yield of squalene did not change significantly. In our study the use of 5% ethanol increased squalene yield at 35 MPa and 40 °C by 13%, up to 0.305 g/100 g seeds. These findings show that polar solvent only slightly enhanced the extractability of

squalene by SC-CO_2, which is quite reasonable for such lipophilic compounds as hydrocarbons.

Extraction and determination of tocopherols

HPLC analysis for tocopherols separation was applied by using reversed-phase, which is based on the structure of side chain and the number of methyl substituents. It should be noted that it was difficult to obtain full separation of β-T and γ-T. The linear relationship between peak area and various concentrations of tocopherols were evaluated and calibration curves demonstrated good linear relationships: α-T, R^2=0.995; β-T, R^2=0.969; γ-T, R^2=0.952; δ-T, R^2=0.994. It may be observed that the oil of amaranth seeds analyzed in this study contained comparatively high amounts of tocopherols (Table 4.13); the total amount of extracted tocopherols was from 112.0 to 317.3 mg/kg seeds and from 5563 to 8178 mg/kg oil. Tocopherol isomers in amaranth oil were distributed at the approximate ratio of 1(α-T):27(β-T):6.5(γ-T):5(δ-T). The highest content of tocopherols, 317.3 mg/kg seeds was achieved at 55 MPa and by adding 5% of cosolvent. In general, SC-CO_2 modified with 5% of ethanol gave higher yields of tocopherols than in case of pure CO_2. The content of extracted tocopherols significantly increased by raising the pressure (p<0.05). This finding could be explained by the modification of solvent polarity when a cosolvent is added and thus better solubilisation of tocopherols was achieved. Total content of tocopherols isolated by SC-CO_2 at the pressures higher than 35 MPa were higher comparing with Soxhlet extraction. These results are in agreement with previously obtained by Bruni et al. (2002), who found that SCE-CO_2 gave quantitatively better yields of tocopherols in shorter times comparing with extraction by organic solvents; the highest tocopherols yield in their study was 129.27 mg/kg seeds, which is approximately up to two times lower compared to our results under similar conditions (230-251 mg/kg seeds) and almost 2.5 times lower comparing with the highest yield of tocopherols (317.29 mg/kg seeds). There were also differences in the distribution between individual compounds; in our study at similar conditions the yields of β-T and γ-T was higher up to 4 and 22 times, respectively, while the yield of α-T and δ-T was lower up to 6 and 1.5 times, respectively. Czaplicki et al. (2011) compared the yields of tocopherols obtained by pressing, organic solvents and SCE-CO_2 and also found that the latter method resulted in the highest yield of tocopherols (131.7 mg/100 g oil). According to León-Camacho et al. (2001), the oil of *A. cruentus* contained only three isomers of tocopherol, namely α-T (248 ppm); β-T (546 ppm) and δ-T (8 ppm). It was also shown that enrichment of tocopherols from double low rapeseed deodorizer distillates can be achieved by SCE-CO_2 and the addition of cosolvent improved the solubility of tocopherols: the best result was obtained in case of 4% cosolvent addition, followed by 2% and 6% (Quancheng, et al., 2004).

Table 4.13. The yield of tocopherols (mg/kg seeds) isolated from amaranth at different extraction conditions

Pressure, MPa	α-T	β-T	γ-T	δ-T	Total tocopherols
Without cosolvent[z]					
15	2.37±0.06[a]	82.42±5.77[a]	12.36±0.18[a]	14.89±0.44[a]	112.0
25	4.92±0.05[b]	129.3±1.25[c]	28.38±0.42[c]	22.95±0.78[c]	185.6
35	5.87±0.07[d]	173.6±1.53[b]	40.93±0.15[b]	31.34±0.48[b]	251.7
45	5.48±0.09[c]	158.9±1.29[d]	36.57±0.32[d]	29.78±0.13[d]	230.7
55	6.31±0.06[e]	187.7±2.29[e]	43.55±0.26[e]	35.73±0.20[e]	273.3
65	6.57±0.18[f]	170.1±1.13[b]	41.21±0.29[b]	31.19±0.73[b]	249.0
2% cosolvent					
15	4.18±0.04[c]	110.0±7.29[a]	23.53±0.52[a]	20.19±0.14[a]	157.9
25	3.57±0.15[a]	124.1±1.23[c]	26.28±1.12[d]	22.79±0.23[c]	176.8
35	6.17±0.18[d]	187.1±2.79[b]	45.81±0.22[bc]	32.91±0.41[b]	272.0
45	6.89±0.31[b]	175.8±1.56[d]	41.48±1.11[e]	32.51±0.11[b]	256.7
55	6.87±0.12[b]	185.9±1.59[b]	46.86±0.85[c]	33.50±0.12[d]	273.1
65	7.54±0.19[e]	194.6±2.42[e]	46.59±0.91[bc]	35.35±0.21[e]	284.1
5% cosolvent					
15	5.63±0.11[a]	157.9±1.18[a]	35.45±0.13[a]	29.16±0.17[a]	228.1
25	5.83±0.13[a]	158.6±1.86[a]	39.32±0.96[b]	28.77±0.11[a]	232.5
35	8.04±0.11[b]	203.0±1.17[b]	52.39±0.28[b]	35.70±0.21[c]	299.2
45	7.53±0.14[c]	200.1±2.25[b]	49.16±0.17[d]	34.78±0.44[b]	291.6
55	9.79±0.09[d]	211.8±1.79[d]	57.07±0.67[e]	38.59±0.15[e]	317.3
65	8.06±0.04[b]	207.8±2.93[d]	52.32±0.99[b]	36.78±0.29[d]	304.9

[z]Ethanol was used as cosolvent;

Results are expressed as a mean ± standard deviation (n=3); different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$).

Antioxidant activity of SC-CO_2 amaranth extracts

The antioxidant activity of amaranth extracts was assessed by L-ORAC assay. The antioxidant activity of amaranth extracts varied from 64.08 to 257.6 μmol TE/g oil (Table 4.14) and it is evident that the extracts obtained with cosolvent possessed significantly higher antioxidant activity comparing with the samples isolated with pure SC-CO_2. This could be explained by the increased polarity of the mixture and consequently better solubility of polar amaranth seed compounds in supercritical fluid. The highest antioxidant activity (257.62 μmol TE/g oil) was obtained for oil samples extracted at 15 MPa with 2% cosolvent and containing the highest concentration of squalene; however the highest amount of antioxidatively active compounds from 1 g of seeds was extracted at 55 MPa with 5% cosolvent (9.96 μmol TE/g). It should be noted that antioxidant activity of extract isolated with pure SC-CO_2 and containing the highest concentration of squalene (Table 4.12) was almost as high as that of the extracts obtained with cosolvent at the same pressure. It indicates that squalene is an important antioxidant in amaranth SC-CO_2 extracts. However, other compounds, most likely tocopherols, are also important contributors to the overall antioxidant activity, because the presence of solely squalene cannot explain high values obtained for the extracts, particularly those which were isolated with the use of a cosolvent. It may be suggested that antioxidant activity of

extracts depends on the concentration of squalene, tocopherols and polar constituents, which are better isolated at higher pressures and by increasing the content of polar cosolvent.

Table 4.14. Antioxidant activity of amaranth extracts evaluated by L-ORAC method and expressed in μmol TE/g of oil or seeds.

Pressure,	without cosolvent[z]		2% cosolvent		5% cosolvent	
MPa	oil	seeds	oil	seeds	oil	seeds
15	235.1±5.21^{c}	3.22	257.6±2.52^{e}	5.51	245.3±7.26^{d}	7.85
25	99.6±4.12^{b}	3.23	169.8±5.43^{b}	5.21	234.3±6.81^{d}	9.79
35	94.1±1.26^{b}	3.54	184.9±3.24^{c}	8.54	186.0±9.11^{b}	9.43
45	64.1±6.24^{a}	2.38	199.6±1.19^{d}	8.86	160.9±2.73^{a}	8.08
55	64.1±2.87^{a}	2.80	155.0±4.81^{a}	7.35	194.6±5.92^{b}	9.96
65	101.8±5.71^{b}	4.07	166.5±1.72^{b}	7.84	171.7±6.74^{a}	8.56
Pure squalene: 304.3±6.81 μmol TE/g						

[z]Ethanol was used as cosolvent

Results are expressed as a mean ± standard (n=3); different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$)

Tikekar et al. (2008) analysed antioxidant activity of amaranth flour lipids extracted by chloroform by ORAC method. The ORAC values of squalene and amaranth flour reported in this study were only 0.74 and 0.14 μmol TE/g respectively and therefore the authors concluded that contribution of squalene to antioxidant activity of amaranth flour was negligible. In this study the lipophilic extracts of amaranth showed higher antioxidant activity as compared to the contribution, which may be expected from the squalene present in the extracts; however, as it was already noted, the extract with high squalene content possessed remarkably higher ORAC values in case of pure SC-CO_2 and 2% cosolvent at 15 MPa. Milan-Carrillo et al. (2012) determined ORAC values for free and bound hydrophilic antioxidants of amaranth seeds and reported that the total antioxidant capacity was 3475 μmol TE/100 g flour. In another study the $ORAC_{FL}$ values of 1 g lycopene depending on its concentration in the assay were reported in the range of approximately 50-150 μmol TE, which is of the same order as for squalene determined in our study (Bangalore, et al., 2005). Considering that lycopene is a branch unsaturated hydrocarbon somewhat similar in the structure to squalene, the ORAC values obtained for this carotene provide the confidence in our results determined for pure squalene.

Fractionation of tocopherols and squalene in separators

The solubility of a target compound in the supercritical fluid which depends on its temperature and density is a major factor in determining its extraction efficiency. Therefore, selection of a proper solvent density is the crucial factor influencing its power and selectivity. In this study the pressure in extraction system was gradually reduced to atmospheric in order to obtain the fraction enriched with tocopherols. Extraction pressure was 55 MPa, temperature 40 °C, extraction time 180 min and flow rate 3 SL/min, i.e. the parameters, which gave the highest yield of tocopherols, 317.3 mg/kg seeds and 6197 mg/kg oil and squalene, 0.296 g/100 g seeds and 5.78 g/100 g oil.

It may be observed that the highest oil content was deposited in the pressure range 25 - 15 MPa because of decreased fluid solvent power (Fig. 4.9). The highest tocopherols recoveries were obtained in the third fractions of the experiments in the pressure range below 15 MPa, although it was failed to separate tocopherols fraction in one of the separators. The major part of squalene was also deposited in the last fraction at atmospheric pressure. The best separation of squalene was obtained in the last experiment resulting squalene recovery up to 17.9 g/100 g oil, first and second fractions contained 2.0 and 2.2 g/100 g oil of squalene at pressure 25 and 15 MPa respectively.

Tocopherol and squalene enriched fraction was previously obtained from fresh palm-pressed mesocarp fibber at 10 MPa compared to 20 and 30 MPa, while carotenes were better extracted at higher pressures (Lau, et al., 2008). These findings are somewhat in agreement with our results obtained by using gradual decrease of pressure in the separators. In another study two separators were used for the fractionation of rice bran oil and it was found that the highest content of tocopherols (24.32 mg/100 g oil) was obtained in the second separator at 2.5 MPa, whereas the fraction precipitating in the first separator, operating at 15 MPa and 25 °C, was 9 times lower in the concentration of tocols (Sarmento, et al., 2006).

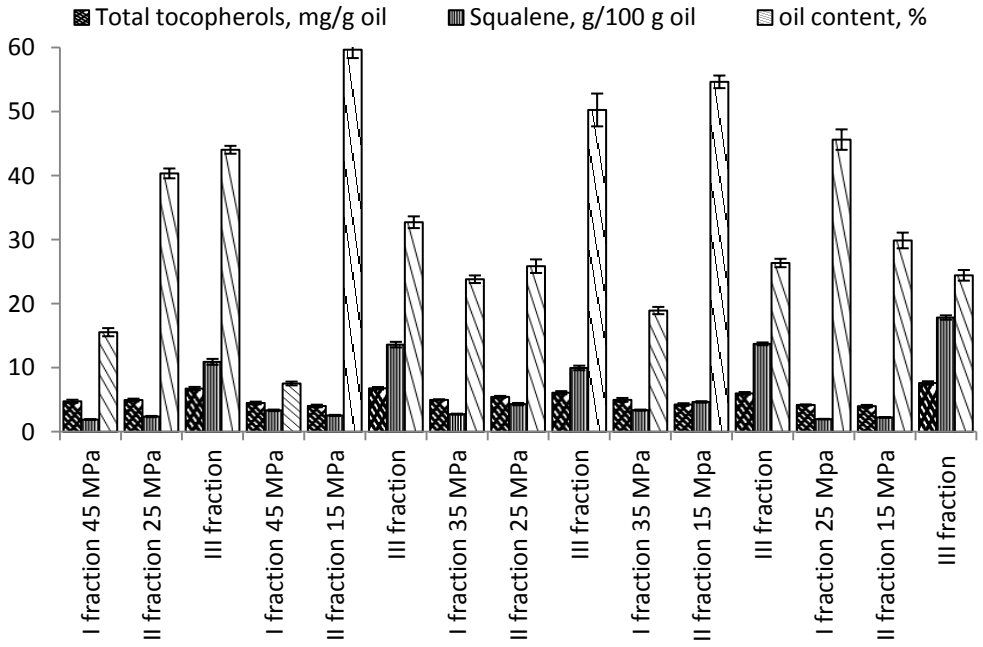


Figure 4.9. Oil content and the recovery of tocopherols and squalene in amaranth oil fractions

Scale-up study

Scale-up study was performed with analytical scale (AS) 0.5 L and pilote scale (PS) 10 L volume extraction vessels. OEC determined by 0.5 L extraction vessel was used as reference to determine pilot scale extraction process duration (Fig. 4.10). The highest amount of oil was obtained during constant extraction rate period (CERP) in 110 min, which accounts for more than 93% of total extract. A diffusion controlled rate period (DCRP) follows by falling extraction rate period (FERP) with a decrease in the extraction rate. The kinetic parameters calculated from the adjustment of AS OEC were t_{CERP}=110 min, $M_{CERP}=6.23\times10^{-5}$ kg/s, R_{CERP}=3.84 % where t_{CERP} is the time of CERP, M_{CERP} is mass-transfer rate and R_{CERP} is the yield of extract during CERP. The value of t_{CERP} represents the minimum time of SFE to keep the process economically viable (Meireles & Angela, 2007)

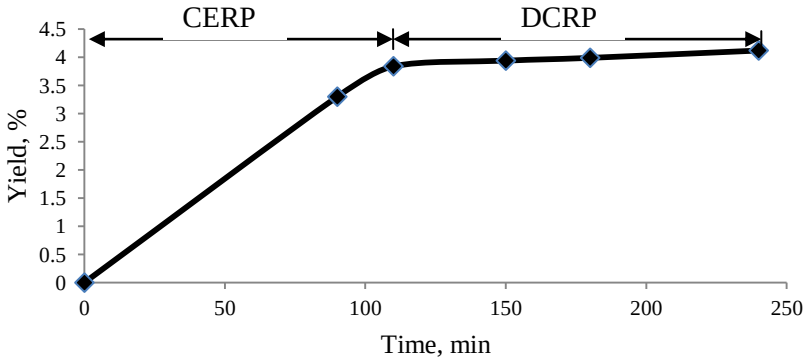


Figure. 4.10. OEC of analytical scale extraction

Amaranth oil yield after 240 min extraction was 4.12 g/100 g seeds (Fig. 4.10). Considering economical aspects, the time of 180 min providing the yield of 97-98% of the highest extractable amount was used in further experiments with 0.5 L volume extraction vessel as well as 10 L volume extraction vessel. The yield of PS was not determined. A 41-fold scale up of amaranth seeds from AS to PS was achieved.

According to Prado et al. (2012), scale-up studies for grape seed revealed that the simple criterion used (constant S/F) was successful in reproducing kinetic behaviour at different scales. These results were in agreement with other scale-up studies. Del Valle et al. (2004) concluded that a simple criterion should be considered with caution, although the simple criterion adopted proved to be more efficient than many complex models proposed in the literature for SFE scale-up.

Chemical composition and antioxidative properties of lipophilic extracts of amaranth isolated with pilot scale extractor

The fractions of the PS experiment collected in separators were analyzed individually. Fig. 4.11 presents squalene content in two fractions isolated gradually decreasing pressure. The highest content of squalene was isolated in the second fraction at pressure range 10-5 MPa. These results are in agreement with my previous studies by

0.5 L extractor, from these results could be concluded that squalene is the best soluble in SC-CO_2 at pressure below 10 MPa. Although still over 12% of squalene left in the remaining fraction, this could be mainly due to limitation of equipment.

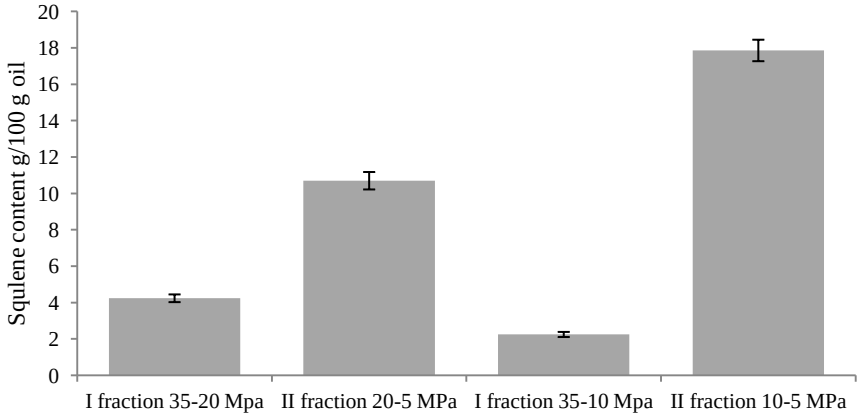


Figure 4.11. Squalene content of amaranth oil fractions

The antioxidant activity of lipophilic fractions was assessed by L-ORAC assay (Fig. 4.12). As could be expected second fraction also possessed the highest activity, mainly due to present of squalene and other active micro constituents as tocopherols. The highest antioxidant activity was 205 μM TE/g oil, similar activity was obtained of the lipophilic extract isolated at 15 MPa (Table 4.12). Results show that fractions obtained at lower pressure contains higher concentration of antioxidant activity possessing substances.

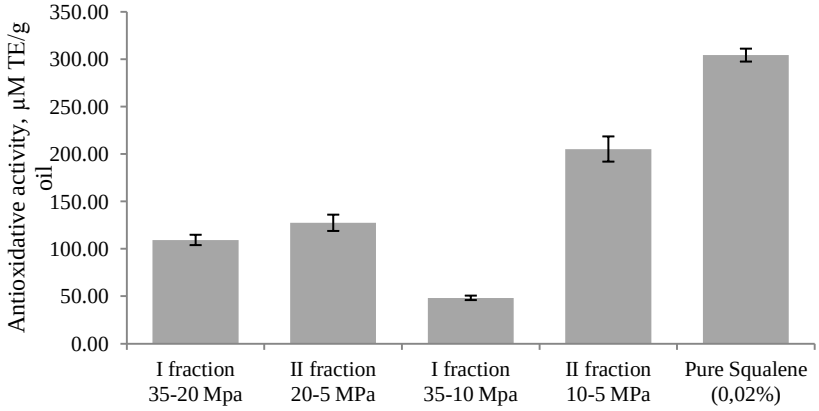


Figure 4.12. Antioxidant activity of amaranth oil fractions and pure squalene

Oil stability was analysed using accelerated oil stability method by Oxipres apparatus. Oil oxidation was accelerated heating oil at 120 °C and pressurised with oxygen at 0.5 MPa. Oxidative oil stability between different fractions was slightly higher in second fraction, although difference was not significant (Fig. 4.13). These results agree with the previous experiments regarding chemical composition and antioxidative activity. In comparison oil stability of amaranth and rapeseed was of the same order. Acording to

Gruzdienė (2007) oil of amaranth possessed 6 times higher oxidative stability than flax seed oil analyzing with Oxipres apparatus at 110 °C.

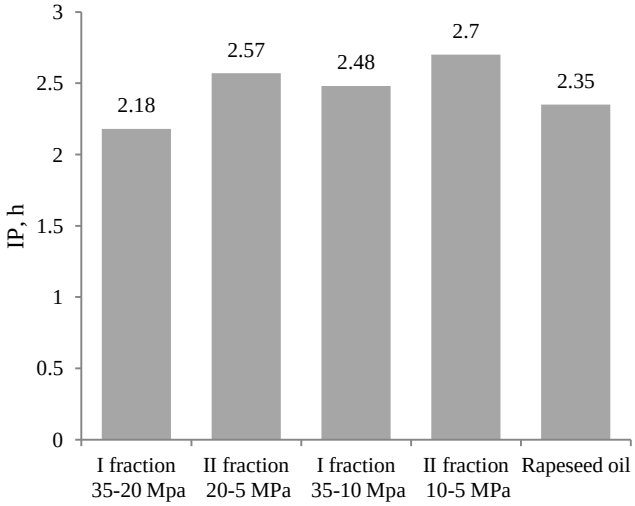


Figure 4.13. Oxidative stability of amaranth oil fractions and rapeseed oil

4.4. Antioxidant properties and preliminary evaluation of phytochemical composition of different anatomical parts of amaranth

Antioxidant activity of amaranth extracts

There are many assays for the assessment of antioxidant properties of plant extracts, the majority of them are based on electron/hydrogen atom transfer reactions. Huang et al. (2005) concluded that ORAC, TPC and one of the electron/hydrogen transfer assays should be recommended for representative evaluation of antioxidant properties of foods. $DPPH^{\bullet}$ assay is mainly attributed to the electron transfer assays, however the quenching of $DPPH^{\bullet}$ radical to form DPPH-H is also possible. Other electron transfer based methods include the total phenols assay by Folin-Ciocalteu reagent and $ABTS^{\bullet+}$ decolourisation assay. Following the above mentioned recommendation all these methods were applied for the assessment antioxidant potential of amaranth in our study.

Acetone extracts were obtained in remarkably (16-77 times) lower amounts comparing to methanol/water extracts (Table 4.15). It indicates that high polarity compounds are dominant in leaves, flowers and stems after removing their lipophilic fraction with hexane. The leaves contained the highest amount of total soluble compounds isolated with both solvents (20.2 g/100 g DW), followed by flowers (16.46 g/100 g DW), stems (15.34 g/100 g DW) and seeds (9.42 g/100 g DW).

The antioxidant properties were measured for extracts and also calculated for the initial dried amaranth material (Table 4.15). Both values provide important information, because amaranth may be used for the isolation of bioactive compounds or as a raw material for cooking as well as the ingredient in various foods. There were remarkable variations in the obtained values between different anatomical parts of amaranth, applied solvent and assay procedure. Comparing the extracts it may be observed that the

differences between RSC of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, ORAC and TPC of flowers and leaves were not remarkable for acetone and methanol/water extracts, although in some cases they were significantly different ($p<0.05$), while the extracts from stems and seeds were remarkably weaker radical scavengers and antioxidants except for ORAC value of SA which was similar to that of LA.

Table 4.15. The yields and antioxidant characteristics of different amaranth anatomical parts extracted by organic solvents $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC expressed in μmol TE/g extracts and μmol TE/g DW plant material); TPC expressed in mg GAE/g extract and mg GAE/g DW plant material)

Samples	Yield	$ABTS^{\bullet+}$		$DPPH^{\bullet}$		ORAC		TPC	
	% w/w	Extract	DW	Extract	DW	Extract	DW	Extract	DW
FA	0.76 ± 0.04^{ab}	334.5 ± 5.28^{d}	2.5^{bc}	28.2 ± 0.48^{b}	0.21^{a}	32.9 ± 0.18^{e}	0.25^{ab}	27.3 ± 1.31^{g}	0.21^{b}
FM	15.7 ± 0.6^{e}	406.4 ± 5.02^{f}	64.0^{e}	50.7 ± 0.68^{g}	7.98^{d}	47.0 ± 0.71^{g}	7.40^{d}	33.3 ± 1.86^{d}	5.2^{e}
LA	1.2 ± 0.08^{b}	336.0 ± 5.14^{d}	4.0^{c}	28.6 ± 0.9^{b}	0.33^{a}	24.7 ± 0.89^{b}	0.29^{ab}	24.8 ± 1.97^{f}	0.29^{b}
LM	19.0 ± 0.93^{f}	395.3 ± 9.37^{e}	75.1^{f}	46.9 ± 0.55^{f}	8.91^{e}	41.4 ± 1.26^{f}	7.86^{d}	32.3 ± 1.81^{d}	6.1^{f}
SA	0.64 ± 0.05^{ab}	47.2 ± 3.68^{c}	0.30^{ab}	16.2 ± 0.12^{e}	0.1^{a}	24.5 ± 0.89^{b}	0.16^{a}	9.2 ± 0.65^{d}	0.06^{a}
SM	14.7 ± 0.85^{d}	40.5 ± 2.47^{b}	5.94^{d}	10.7 ± 0.52^{c}	1.57^{c}	4.79 ± 1.28^{c}	0.70^{b}	7.3 ± 0.41^{a}	1.1^{d}
SDA	0.12 ± 0.01^{a}	32.1 ± 0.93^{a}	0.03^{a}	14.6 ± 0.65^{d}	0.01^{a}	15.3 ± 0.89^{d}	0.02^{a}	10.6 ± 0.34^{e}	0.01^{a}
SDM	9.3 ± 0.44^{c}	30.1 ± 1.54^{a}	2.79^{d}	9.5 ± 0.73^{a}	0.88^{b}	4.0 ± 0.39^{a}	0.37^{ab}	8.1 ± 0.33^{ab}	0.8^{c}

Values represented as mean ± standard deviation (n=3);
Different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$).

The samples of plant materials showed significant differences in antioxidant activities between the extracts isolated with different solvents ($p<0.05$). FA and LA were weaker antioxidants than FM and LM, whereas SA and SDA possessed stronger RSC. For instance, the highest RSC of FM in $ABTS^{\bullet+}$ assay was 406.4 μmol TE/g, that is 1.2 times higher than for FA; in $DPPH^{\bullet}$ assay it was 50.7 μmol TE/g and in ORAC 47.0 μmol TE/g. In general, the values in $DPPH^{\bullet}$ assay were remarkably lower comparing to $ABTS^{\bullet+}$ assay. It may be explained by different reaction kinetics and the peculiarities of the used reagents. It is also interesting to note that the differences between these values are bigger when antioxidant capacity values are higher. A strong correlation between TPC and antioxidant activity was observed: TPC vs. $ABTS^{\bullet+}$, $R^2 = 0.99$: TPC vs. $DPPH^{\bullet}$, $R^2 = 0.998$ and TPC vs. ORAC, $R^2 = 0.988$). Thus, the TPC is a good predictor of the *in vitro* antioxidant activity for amaranth extracts.

The values of RSC and antioxidant activity in plant dry material highly depend on extract yield. Actually, these values show how much of TE antioxidants can be isolated with the selected solvent from 1 g of plant DW. So far as the yields of polar extracts were remarkably higher than those of acetone extracts, the total content of isolated antioxidants from 1 g of plant DW by methanol/water was many times higher. The sum of the values obtained for both solvents may be considered as a total amount of TEs present in 1 g of DW plant material. However, it should be noted that hexane which usually extracts very low amount of antioxidants from leafy vegetables, effectively isolates abundant lipohilic fraction from seeds, containing lipid soluble antioxidants, such as tocols and squalene.

As it was mentioned, antioxidant properties of amaranth seeds and leafy parts were studied previously by using different methods, however the results obtained are difficult

to compare. For instance, Paśko et al. (2009) compared total antioxidant capacity of two amaranth species with quinoa seeds and sprouts and determined that the seed extract of amaranth possessed lower antioxidant activity than quinoa, whereas the TPC in the whole (not defatted) amaranth seeds in their study was up to 4 times higher comparing to our results. The antioxidant capacities determined by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays and estimated as TEAC interpolated to 50 % inhibition values ($TEAC_{50}$) in sprout extracts of amaranth (Paśko, et al., 2009) were up to 2 times lower comparing with our results on amaranth leaves. Nsimba et al. (2008) evaluated antioxidant capacity and TPC in defatted amaranth seeds and reported that the highest antioxidant capacity possessed *A. cruentus*, which scavenged 85.6 % of $DPPH^{\bullet}$ at the applied concentration. The highest TPC of 133.2 mg/g tannic acid equivalents was found for *A. hypochondriacus* seeds and it is remarkably higher as compared with the defatted seeds analysed in our study. Oboh et al. (2008) reported that polar extracts obtained by soaking *A. cruentus* leaves in water for about 24 hours possessed 1.4 times higher TPC than LM analyzed in our study.

Direct evaluation of antioxidant capacity by QUENCHER method

Some antioxidatively active constituents may be strongly bound to other components in plant material matrix and are not extracted by organic solvents or water. Such compounds may be released in human intestinal tract during digestion. Recently a QUENCHER method was developed, which determines the antioxidant activity of the whole plant material including its insoluble fraction. According to Serpen et al. (2007), the values obtained by using QUENCHER method for insoluble food components show a significant antioxidant activity, which in many cases remarkably higher than those obtained by the traditional extraction procedures. They hypothesized that free functional groups on the surface of insoluble particles quench with radicals.

It may be observed that all QUENCHER values for the leaves were higher than for other parts, except for $DPPH^{\bullet}$, when RSC of leaves and flowers were similar (Table 4.16). The RSC and antioxidant activity of stems and seeds were remarkably lower, which is in agreement with the results obtained for the extracts. However, comparing QUENCHER results with those obtained by analyzing the extracts some interesting observations can be noticed. The sum of RSC of leaves and flowers obtained in $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assay by analyzing the extracts isolated by both solvents and calculated for 1 g of DW was approximately 2 times lower than the relevant values obtained by a QUENCHER assay. This difference for seeds was even higher. The sum of values obtained in ORAC assay by analyzing the extracts isolated by both solvents and calculated for 1 g of DW was even more times higher in case of QUENCHER assay; however the differences in TPC values were less remarkable, except for the seeds. Several reasons may be raised to explain these differences. First of all, as it was already mentioned, some part of antioxidatively active compounds may remain in the matrix after extraction because they are bound to other constituents. Some classes of compounds, e.g antioxidatively active proteins and carbohydrates may be insoluble in the used solvents while in QUENCHER assay their active sites in the structures may participate in antioxidant processes. Finally, the systems of assay are different and the differences in the assay matrix may influence reaction kinetics. In $DPPH^{\bullet}$ assay, on the contrary to other methods, the seeds were stronger

radical scavengers than stems. The defatted seeds contain high amount of proteins and some of them may be antioxidatively active substances.

Table 4.16. Antioxidant activity (μmol TE/g DW) and total phenols content (mg GAE/kg DW) of amaranth parts obtained by QUENCHER procedure

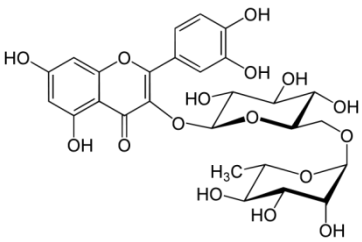
Samples	$ABTS^{\bullet+}$	$DPPH^{\bullet}$	ORAC	TPC
Flowers	112.33±7.45[b]	16.73±0.35[c]	35.5±0.71[c]	7.86±0.02[c]
Leaves	144.24±2.41[c]	16.42±0.37[c]	51.3±3.73	10.08±0.02[d]
Stems	19.05±1.13[a]	1.72±0.02[b]	12.2±0.31[a]	1.73±0.02[a]
Seeds	21.82±1.06[a]	3.96±0.03[a]	15.7±1.35[b]	3.70±0.02[b]

Values represented as mean ± standard deviation (n=3);
Different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$).

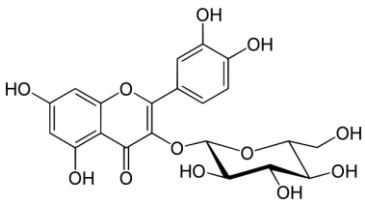
A strong correlation between TPC and antioxidant activity measured by a QUENCHER method was also observed: TPC vs. $ABTS^{\bullet+}$, $R^2 = 0.95$; TPC vs. ORAC, $R^2 = 0.97$. To the best of our knowledge no results have ever been published on evaluating amaranth seeds and leaves using QUENCHER method.

Preliminary characterization of amaranth phytochemicals by chromatographic analysis

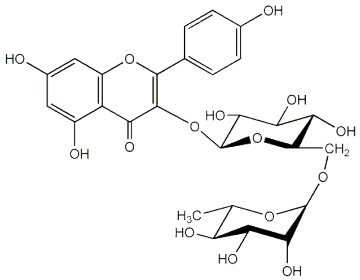
A reversed-phase HPLC separation coupled with PDA detector and the $DPPH^{\bullet}$ scavenging detector was used for the preliminary screening of antioxidant compounds which may be present in different amaranth anatomical parts.



Rutin



Isoquercitin



Nicotiflorin

Figure 4.14. The main glycosides of amaranth leaves and flowers

In total, 5 compounds were identified by comparing their spectral properties with literature data (Fig. 4.14). Rutin is dominating compound in all extracts of amaranth

anatomical parts. The typical chromatograms of amaranth FM extracts are shown in Fig. 4.15, the intensities of peaks in the chromatograms is given in Table 4.17. Its negative peak in chromatogram was the major indicating that rutin was the main constituent in amaranth leaves scavenging DPPH$^{\bullet}$. Rutin as the main amaranth flavonoid was reported previously; the content of this compound in amaranth varied from 0.08 (seeds) to 24.5 g/kg DW (leaves) (Kalinova & Dadakova, 2009).

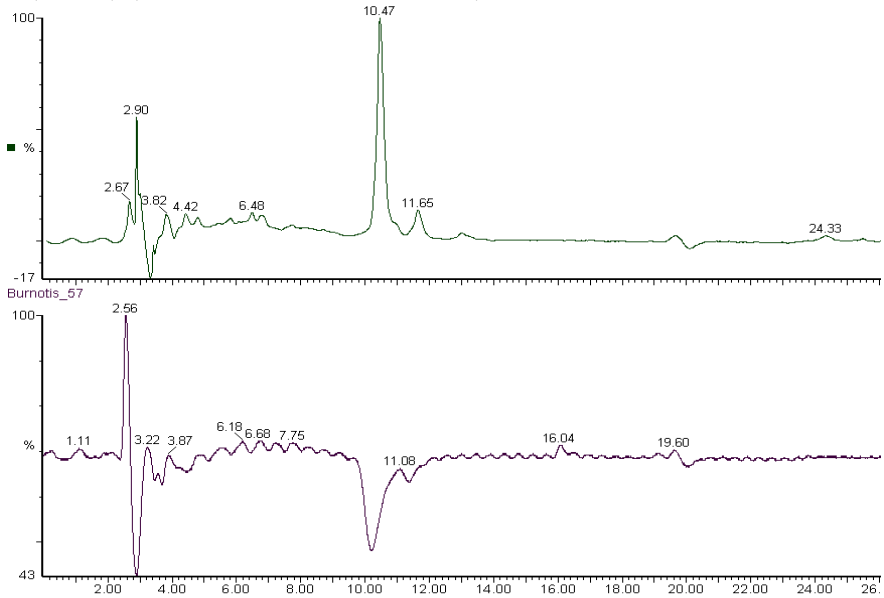


Figure 4.15. Typical chromatograms of amaranth flower methanol:water extracts HPLC-UV-DPPH$^{\bullet}$

Table 4.17. Peak areas (arbitrary units) of identified constituents in different amaranth extracts

Extract	4-Hydroxybeizoic acid	Rutin	Isoquercitrin	Nicotiflorin
Flowers M	-	192243	23213	6088
Flowers A	4634	63255	16506	4434
Seeds M	-	-	-	-
Seeds A	3648	2610	-	-
Leaves M	-	121950	4975	6309
Leaves A	2408	57317	3460	6004
Stems M	-	traces	1117	-
Stems A	14300	traces	3467	-

For preliminary screening of extracts they were analysed by UPLC-QTOF-MS. The chromatogram of amaranth LA is shown in Fig. 4.16. In total, 5 compounds were identified by comparing their spectral properties with literature data. Peak **1** had a retention time of 0.49 min, its MS gave m/z=163.0403, corresponding to ion formula $C_9H_7O_3$. So far as the peak of this compound was overlapping with other eluting from the column peaks, the extraction of UV data was complicated. Based on its accurate mass compound **1** was identified as *p*-coumaric acid. Peak **2** had a retention time of 2.18 min

and gave MS peak at m/z = 137.0246, corresponding to ion formula $C_7H_5O_3$. UV spectral data for this compound was also not available due to small concentrations and overlapping with other peaks. This compound was identified as 4-hydroxybenzoic acid. Peak **3** had a retention time 5.42 min and gave an m/z = 609.1462, corresponding to ion formula $C_{27}H_{29}O_{16}$. This compound had UV maxima at 254, 265 (sh) and 354 nm. Based on this data and by comparing it with the standard, this compound was identified as rutin. Peak **4** had a retention time 5.65 min and m/z value 463.0886, corresponding to ion formula $C_{21}H_{19}O_{12}$. Its UV spectrum had maxima at 254, 266 (sh) and 354 nm, corresponding to quercetin aglycone moiety. This compound was identified as isoquercitrin. Peak **5** had a retention time 6.24 min and m/z value of 593.1518. A ion formula of $C_{27}H_{29}O_{15}$ was calculated from this accurate mass, while UV spectral data gave maxima at 167 and 237 nm. Based on literature data this compound was tentatively identified as nicotiflorin. Similar compounds were identified in the acetone extracts of plant stems and flowers as well. It may be clearly observed that rutin (peak nr. 3 in Fig. 4.16) is the major quantitatively constituent in amaranth extracts, which is in agreement with other studies on amaranth flavonoids. A large number of recorded peaks on the chromatogram indicate that the extracts are complex mixtures of compounds; however, exact mass and fragmentation data obtained by UPLC-QTOF-MS was not sufficient for their identification, because mass spectra libraries give too many candidate structures for the measured mass. Purification of compounds and analysis by NMR would be necessary for positive identification of amaranth constituents.

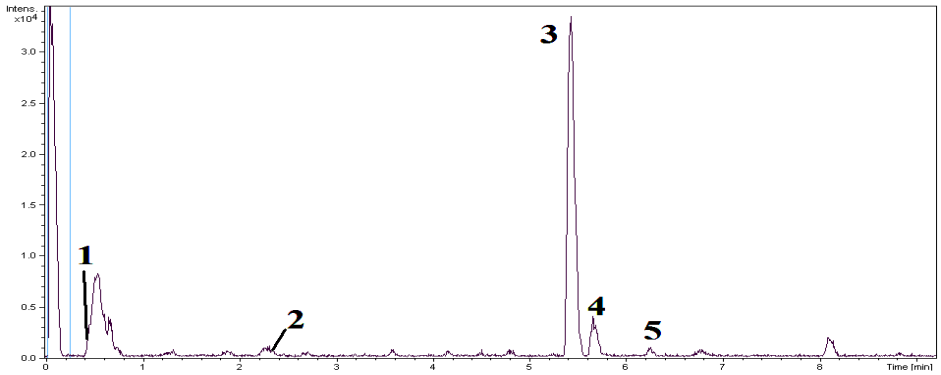


Figure 4.16. Typical chromatograms of amaranth flower methanol:water extracts UPLC-Q-TOF (1- *p*-coumaric acid, 2 - 4-hydroxybenzoic acid, 3 - rutin, 4 - isoquercitrin, 5 - nicotiflorin)

Various phenolic compounds and flavonoids were reported previously in amaranth seeds and vegetables. For instance, the sprouts of amaranth contained rutin as the main constituent and gallic, *p*-coumaric and syringic acids as other important constituents (Paśko, et al., 2008). It was also shown that the content of polyphenols in different amaranth seed varieties were influenced by many factors, such as genotype, climatic and environmental conditions, experimental sites and seasons (Steffensen, et al., 2011). The content of ferulic, caffeic and *p*-coumaric acids in amaranth seed methanol extracts were higher than in quinoa seeds and

soybeans (Oboh, et al., 2008). Rutin, isoquercitrin and nicotiflorin were quantified in amaranth seed flours (Barba de la Rosa, et al., 2009), rutin was also analysed in the methanol extract isolated from the whole plant of *A. spinosus* by using HPTLC plates and it was determined that its concentration in the whole plant powder was 0.15 % (Suryavanshi, et al., 2007).

Table 4.18. Experimental design for the extraction of amaranth leaves

Extraction No.	Temperature, °C	Time, min	H_2O/EtOH, v/v	Total extract yield, g/100 g plant DM	Rutin recovery, mg/kg plant DM
1	124	12	50/50	30.2	13459
2	80	17	80/20	24.4	12478
3	50	12	50/50	19.8	12529
4	124	20	50/50	32.4	14033
5	124	12	50/50	30.0	13111
6	168	17	20/80	30.6	10144
7	124	12	0/100	10.2	6950
8	124	12	100/0	31.3	9898
9	168	8	20/80	27.1	9663
10	80	17	20/80	16.0	10502
11	124	12	50/50	30.7	13205
12	124	5	50/50	26.9	12834
13	80	8	80/20	23.3	11847
14	168	8	80/20	40.5	12724
15	124	12	50/50	30.4	13166
16	80	8	20/80	13.2	9782
17	124	12	50/50	30.2	12815
18	168	17	80/20	44.2	13758
19	198	12	50/50	49.9	13291
20	124	12	50/50	30.0	12803

4.5. Rutin isolation from different anatomical parts of *Amaranthus paniculatus* using accelerated extraction with green solvents

Rutin isolation by pressurised liquid extraction

Response surface methodology (RSM) was used to maximize rutin isolation and extract yield optimizing three independent variables (temperature, extraction time, and water/ethanol ratio) at five levels. The total yield of extract from amaranth leaves varied from 10.2 to 55.8 g/100 g dry mater (DM) and the recovery of rutin varied from 7280 to 14304 mg/kg DM (Table 4.18). The highest total extract yield and rutin content values were obtained under the same extraction conditions: water/ethanol ratio 70:30 (v/v), 188 °C temperature and 20 min static extraction time. In this case maximum rutin yield from the leaves was 4-times higher and the total extraction yield was 10 times higher comparing to that attainable using conventional ultrasonic assisted extraction, using methanol and ethanol mixture as a solvent (Table 4.19). Predicted value under optimal conditions was 14351 mg/kg DM for the rutin recovery, which is not significantly different from the actual value. Rutin content in the defatted and not defatted seeds of

amaranth under optimal conditions was significantly lower, 35.3 and 41.1 mg/kg DM, respectively, while in case of ultrasonic assisted extraction it was 19.7 and 34.6 mg/kg DM, respectively. In my experiments the rutin content obtained from *Amaranthus paniculatus* was similar.

Table 4.19. Comparison of the rutin yield obtained by PLE and ultrasound assisted extraction from amaranth leaves and seeds

Extraction method	Condition	Total extract yield, g/100 g plant DM	Rutin recovery mg/kg DM
	Leaves		
PLE	Optimal conditions: 188 °C, 20 min, 70:30 water/ethanol (v/v)	55.8±0.64	14304
PLE	70 °C, 18 min, 70:30 methanol/water (v/v)	16.4±0.12	11322
Ultrasound assisted extraction	25 °C, 50 min, 90:10 methanol/ethanol (v/v)	5.8±0.07	3621
	Defatted seeds		
PLE	Optimal conditions: 188 °C, 20 min, 70:30 water/ethanol (v/v)	67.9±1.6	35.4
PLE	70 °C, 18 min, 70:30 methanol/water (v/v)	2.1±0.08	17.9
Ultrasound assisted extraction	25 °C, 50 min, 90:10 methanol/ethanol (v/v)	2.8±0.02	19.7
	Not defatted seeds		
PLE	Optimal conditions: 188 °C, 20 min, 70:30 water/ethanol (v/v)	64.2±0.38	41.1
PLE	70 °C, 18 min, 70:30 methanol/water (v/v)	4.7±0.26	49.8
Ultrasound assisted extraction	25 °C, 50 min, 90:10 methanol/ethanol (v/v)	6.5±0.01	34.7

Response surface plots showing the effect of extraction time, temperature and water content in the solvent on rutin yield are presented in Fig. 4.16. The graphs were obtained by fixing one variable at optimal value, while varying the remaining two variables and predicting the response variable. Fig. 4.17 illustrates the linear effect of extraction temperature and time and quadratic effect of water content in the solvent on rutin yield. Water content in the mixture with ethanol had the main influence on rutin yield comparing with the effect of extraction temperature and time. Rutin yield was maximized at water/ethanol ratio 70:30 and the lowest rutin content isolated using 100% ethanol. Temperature and time had a linear and minor effect on rutin isolation. This study also shows that temperature up to 190 has no influence on rutin degradation. Parameters as pressure and time possess less critical effect on extraction rate (Herrero, et al., 2013).

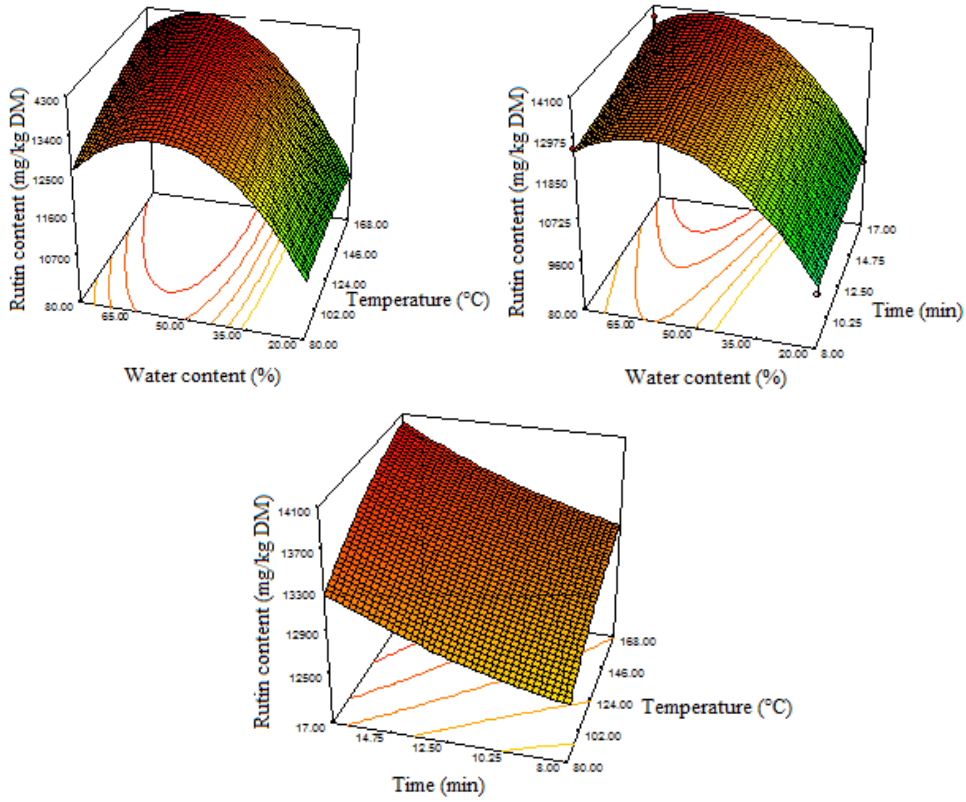


Figure 4.17. Response surface plots showing the effect of extraction time, temperature and water content in the mixture of solvent on rutin content

For instance, Garcia-Marino et al. (2006) showed that by using water in sequential extraction from 50 °C to 200 °C, it was possible to extract different procyanidins according to its degree of polymerization and structure. Increasing temperature favored compounds formed by more than one unit of catechin. In other study, compound class selectivity was observed at different extraction temperatures providing extracts with different phenolic profiles. Results showed that, at the lowest temperature, the most polar compounds were preferentially extracted (flavanones/dihydroflavonol such as dihydroquercetin, eriodictyol, and dihydrokaempferol), whereas at 200 °C, the solubility of less polar compounds was increased by several orders of magnitude (Turner & Ibañez, 2011).

The highest extract amount was isolated at the same conditions as previous Rutin. Response surface plots showing the effect of extraction time, temperature and water content in the solvent on extract yield are presented in Fig. 4.17. Unlike previous CCD experiments the most significant factor influencing extract yield was temperature following water content in the solvent mixture and time, respectively. The highest yield of extract obtained at the highest temperature 200 °C (Fig. 4.18). Herrero et al. (2013) reported that temperature is critically important to the extraction process in terms of extraction yield. It can be observed that the yield is obviously influenced also by water content in the mixture with ethanol. The yield of extract increased increasing water

content in the solvent up to 70%. Changes in the extraction time had low influence on extract yield.

High temperature facilitates faster analyte diffusion and reduces interaction between analytes and the sample matrix by disrupting intermolecular forces as a result increasing the yield of extract. Water at 200°C has a dielectric constant similar to pure methanol at room temperature (Yang, et al., 1998).

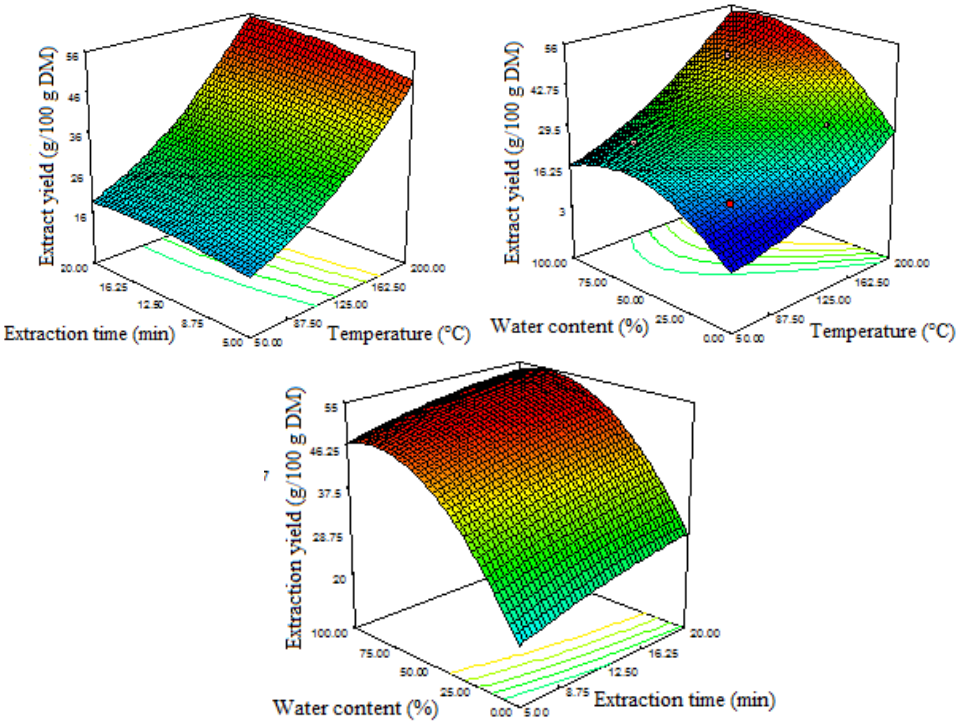


Figure 4.18. Response surface plots showing the effect of extraction time, temperature and water content in the solvent on extract yield

Estimated effects of each factor are shown in Pareto chart in decreasing order of importance (Fig. 4.19). The length of each bar is proportional to the standardized effect, which is the estimated effect divided by its standard error. Bars which extend beyond the line correspond to effects which are statistically significant at the 95.0% confidence level. In this case, 6 effects are significant in decreasing order: temperature (A), water content in the solvent (C), the interaction effect of water content (C^2), extraction time (B), the interaction effect between temperature (A^2) and interaction effect between temperature and water content (A×C).

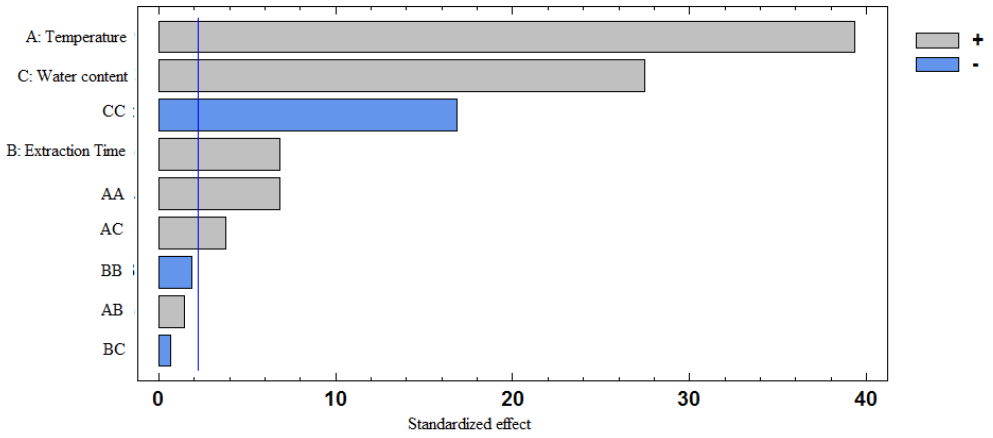


Figure 4.19. Standardized Pareto chart for extract yield

Model fitting

The effect of extraction temperature, time and water content in the mixture of the solvent on total extract and rutin yield obtained by PLE was investigated using response surface CCD. The results were analysed by using Analysis of variance (ANOVA). The model for rutin content investigation was significant (Table 4.20). Temperature, time and water content were significant variables. The highest significant effect on rutin content had water content and extraction time ($p<0.01$) and interaction between temperature and water content. Not significant lack of fits means that model fits.

Table 4.20. Analysis of variance for the experimental results of rutin content investigation

Source	*Sum of Squares*	*df*	*Mean Square*	*F-Value*	*p-value Prob > F*	
Model	6.304E+007	9	7.004E+006	68.05	< 0.0001	significant
A-Temperature	*6.422E+005*	*1*	*6.422E+005*	*6.24*	*0.0316*	
B-Time	*1.746E+006*	*1*	*1.746E+006*	*16.96*	*0.0021*	
C-Water content	*1.834E+007*	*1*	*1.834E+007*	*178.16*	*< 0.0001*	
AB	*3362.00*	*1*	*3362.00*	*0.033*	*0.8602*	
AC	*8.672E+005*	*1*	*8.672E+005*	*8.43*	*0.0158*	
BC	*26912.00*	*1*	*26912.00*	*0.26*	*0.6202*	
A^2	*1.160E+005*	*1*	*1.160E+005*	*1.13*	*0.3134*	
B^2	*1.312E+005*	*1*	*1.312E+005*	*1.27*	*0.2853*	
C^2	*4.085E+007*	*1*	*4.085E+007*	*396.88*	*< 0.0001*	
Residual	1.029E+006	10	1.029E+005			
Lack of Fit	*7.158E+005*	*5*	*1.432E+005*	*2.28*	*0.1930*	*not significant*
Pure Error	*3.135E+005*	*5*	*62707.37*			
Total (corr.)	6.407E+007	19				

The R-Squared statistic indicates that the model as fitted explains R^2= 0.9839% of the variability in yield. The adjusted R-squared statistic, which is more suitable for

comparing models with different numbers of independent variables, is R^2(Adj.) =0.9695%. The R^2(Pred.) of 0.9075 is in reasonable agreement with the R^2(Adj.) of 0.9695.

Fitting a regresion surface to the experimental results, the following equation was obtained in order to predict achievable rutin content. The equation of the fitted model is:

$$Y=6848.15+2.65\times A-72.62\times B+190.8\times C+0.1\times A\times B+0.25\times A\times C+0.43\times B\times C-0.05\times A^2+4.71\times B^2-1.88\times C^2 \quad (4.2)$$

Where Y is response (rutin content) and A, B and C are the coded values of factors, temperature, time and water content respectively.

Predicted values were calculated using a second order polynomial equation (3.2) and compared with experimental values.

The ANOVA table of leaves extract yield test the statistical significance of each effect by comparing the mean square against an estimate of the experimental error (Table 4.21). The six effects of the model indicate that they are significantly different ($p<0.05$) from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 99.6% of the variability in Yield. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 99.3%. The standard error of the estimate shows the standard deviation of the residuals to be 0.798.

Table 4.21. Analysis of variance for the experimental results of extract yield from amaranth leaves investigation

Source	*Sum of Squares*	*df*	*Mean Square*	*F-Value*	*p-Value Prob > F*
A-Temperature	987.366	1	987.366	1548.44	< 0.0001
B-Time	30.0136	1	30.0136	47.07	< 0.0001
C-Water content	481.277	1	481.277	754.77	< 0.0001
AB	1.36125	1	1.36125	2.13	0.1747
AC	9.245	1	9.245	14.50	0.0034
BC	0.26645	1	0.26645	0.42	0.5326
A^2	29.6291	1	29.6291	46.47	< 0.0001
B^2	2.21717	1	2.21717	3.48	0.0918
C^2	181.383	1	181.383	284.46	< 0.0001
Total error	6.3765	10	0.63765		
Total (corr.)	1744.31	19			

The equation of the fitted model is:

$$Y = -1.78-0.06\times A-0.62\times B+0.51\times C+0.00074\times A^2+0.0021\times A\times B+0.0008\times A\times C-0.019\times B^2-0.0013\times B\times C-0.0039\times C^2 \quad (4.3)$$

Where Y is response (extract yield) and A, B and C are the coded values of factors, temperature, time and water content respectively.

Predicted values were calculated using a second order polynomial equation (3.2) and compared with experimental values.

Supercritical antisolvent fractionation of rutin

Supercritical antisolvent fractionation has been developed for the fractionation of previous isolated leaves extract at optimal conditions to obtain rutin enriched fraction by antisolvent precipitation. Flavonoids are practically insoluble in pure supercritical CO_2, but sufficient soluble in CO_2 + ethanol (Catchpole, et al., 2004). SC-CO_2 is used as an anti-solvent to precipitate high molecular mass components very polar compounds and as a solvent to extract medium polarity, ethanol soluble components. Feed mixture (leaves extract) was separated into two fractions, according compounds solubility in SC-CO_2; a concentrated flavonoid fraction as the primary product (rafinate) and an ethanol fraction as a secondary product (extract). The effect of pressure and flow rate ratio of feed mixture on rutin recovery was determined. The separation performance depends on the solvent composition hydro-alcoholic solvent extraction process, the ratio of feed solution to supercritical fluid, the feed solids concentration and the pressure used for the antisolvent and separator stages.

Rutin and other the most polar compounds, mostly glycosides, are insoluble in supercritical CO_2 and thus composed rafinate. The extraction and fractionation behaviour was investigated by RSM at following conditions (Table 4.22). The effect of pressure and the ratio of solvent feed to CO_2 were analysed. The highest rutin content was determined at 26 MPa.

Table 4.22. Experimental design for the supercritical antisolvent fractionation

Run	Pressure, MPa	Feed mixture flow rate ml/min	Rutin recovery mg/100 g DM
1	30	0.4	1525
2	30	0.2	1479
3	25	0.16	1584
4	32.1	0.3	1380
5	20	0.2	1626
6	25	0.3	1621
7	25	0.3	1630
8	25	0.3	1600
9	18	0.3	1780
10	20	0.4	1770
11	25	0.3	1609
12	25	0.44	1640
13	25	0.3	1590
14	15	0.3	2257
Non fractionated hydro-alcoholic mixture			1600

The ratio of feed solution to CO_2 cannot be increased indefinitely because decrease separation performance of medium polarity components that are soluble in CO_2+liquid mixture and increase looses of ethanol into rafinate. Higher feed mixture flow rate (up to 0.44 ml/min) showed better rutin recovery in rafinate than lower flow rate at higher

pressure. Ethanol was largely coextracted by CO_2 whereas the amount of water coextraction with CO_2 and ethanol increase with increasing pressure at fixed temperature and accumulated in rafinate. Increasing pressure above 20 MPa some rutin passed to extract fraction. As the ratio of liquid extract and CO_2 increase beyond a certain level, near critical fluid phase becomes sub-critical liquid. The recovery of rutin in rafinate is shown as a function of feed mixture to CO_2 and pressure (Fig. 4.20). Antisolvent pressure was the most significant model factor influencing rutin recovery (p<0.0001) and feed rate had lower effect (p<0.05).

Antisolvent fractionation of propolis tincture in CO_2+ethanol was used to concentrate flavonoids in first fraction and essential oil/ethanol as second product fraction (Catchpole, et al., 2004). Gonzalez-Coloma et al. (2012) presented an updated overview of SFE and SAF of natural products. They compared the results of SFE, SAF and organic soxhlet extraction of *Persea indica*, a Macaronesian paleoendemism with strong insecticidal components.

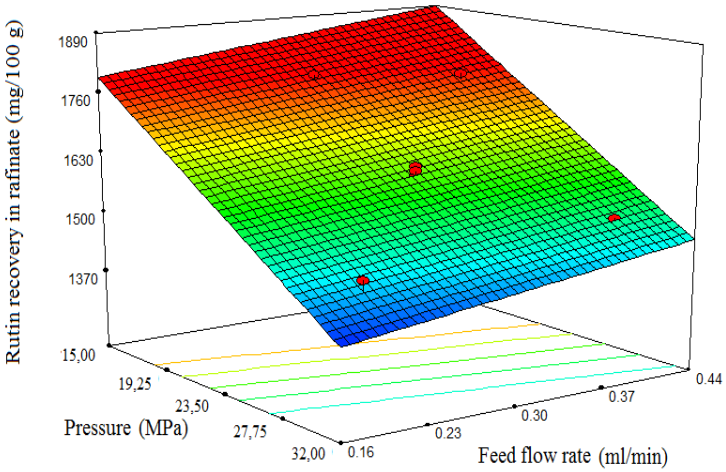


Fig. 4.20. Response surface plot of rutin recovery in rafinate

4.6 Bio-refinery of amaranth anatomical parts

All amaranth anatomical parts could be consumed for human food. Amaranth seed residues after oil extraction are considered as by-product however it is source of high protein amaranth flour or could be separate as protein and starch isolate. The oil in amaranth is present in relatively high amounts (~20%) in the germ fraction which in turn represents around 25% of the weight of the seed (Berganza, et al., 2003). Scheme of seeds bio-refinery presented in fig. 4.21. For example in order to use seeds more rationally, their components can be separated into fractions during grinding and sieving process: fine fraction-containing (14.3%) oil and (25.8%) protein, middle fraction – containing 6.4% oil and 11.2% proteins and coarse fraction – containing up to 1% of oil and protein. The highest part of perisperm contains starch which is very valuable and useful and could be used to enrich other food products. Oil from fine and middle fractions could be extracted and fractionated using supercritical carbon dioxide, obtaining healthy, high value edible

oil fraction enriched with squalene and tocopherols. The residues of seeds after oil extraction are very valuable and contain over 30% of valuable proteins which are very valuable and easily digestible also fine particles contains high amount of mineral and vitamins. The residues of fine particles also could be used to enrich other lower value food products.

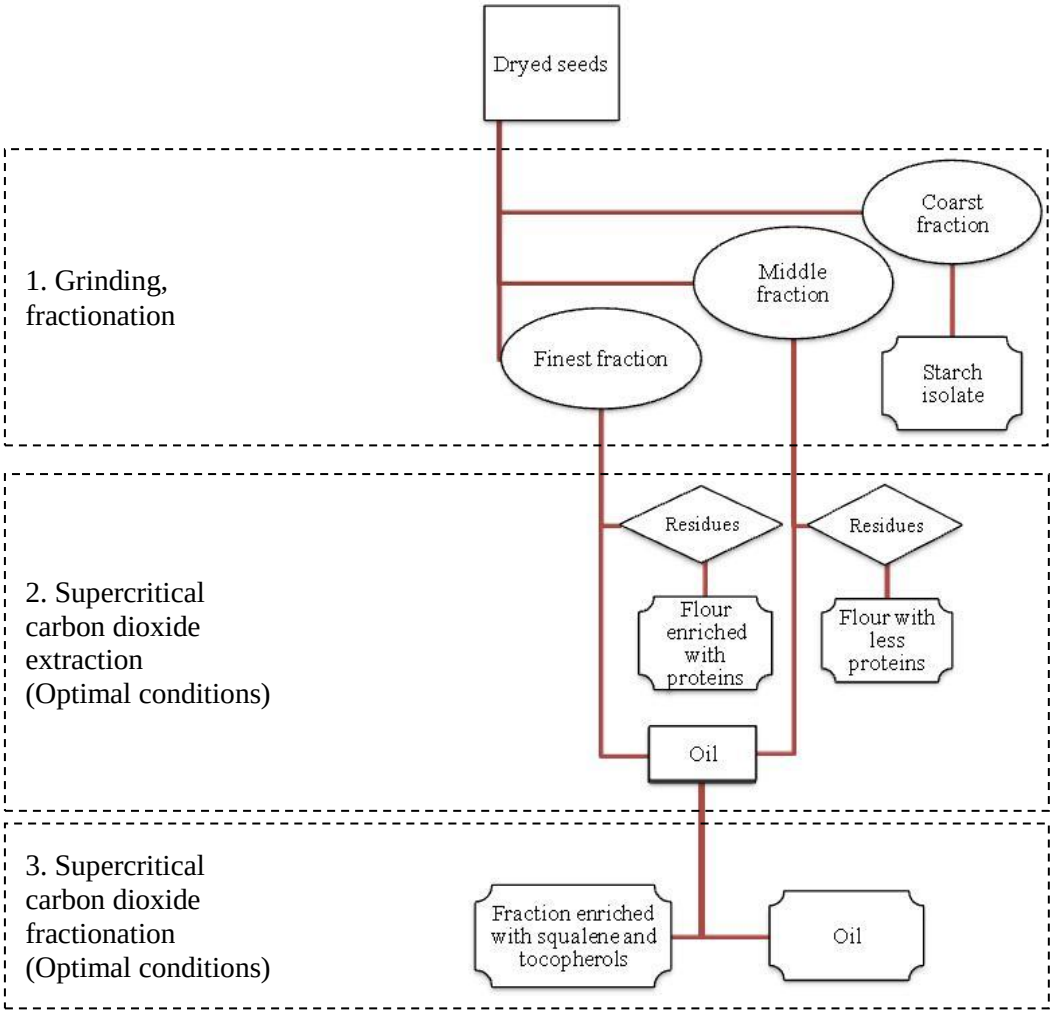


Fig. 4.21 Scheme of bio-refinery of amaranth seeds

Amaranth leaves could be used as fresh for salads or soups. Leafy parts of the amaranth plants contain many times higher amounts of flavonoids than the seeds. Rutin is the main quercetin glycoside present in amaranth leaves. Dried amaranth leaves could be used for subcritical water extraction in order to obtain rutin enriched extracts (Fig. 4.22). Applying complex extraction and fractionation of rutin is possible to obtain extracts containing high amount of rutin (2257 mg/100 g DM).

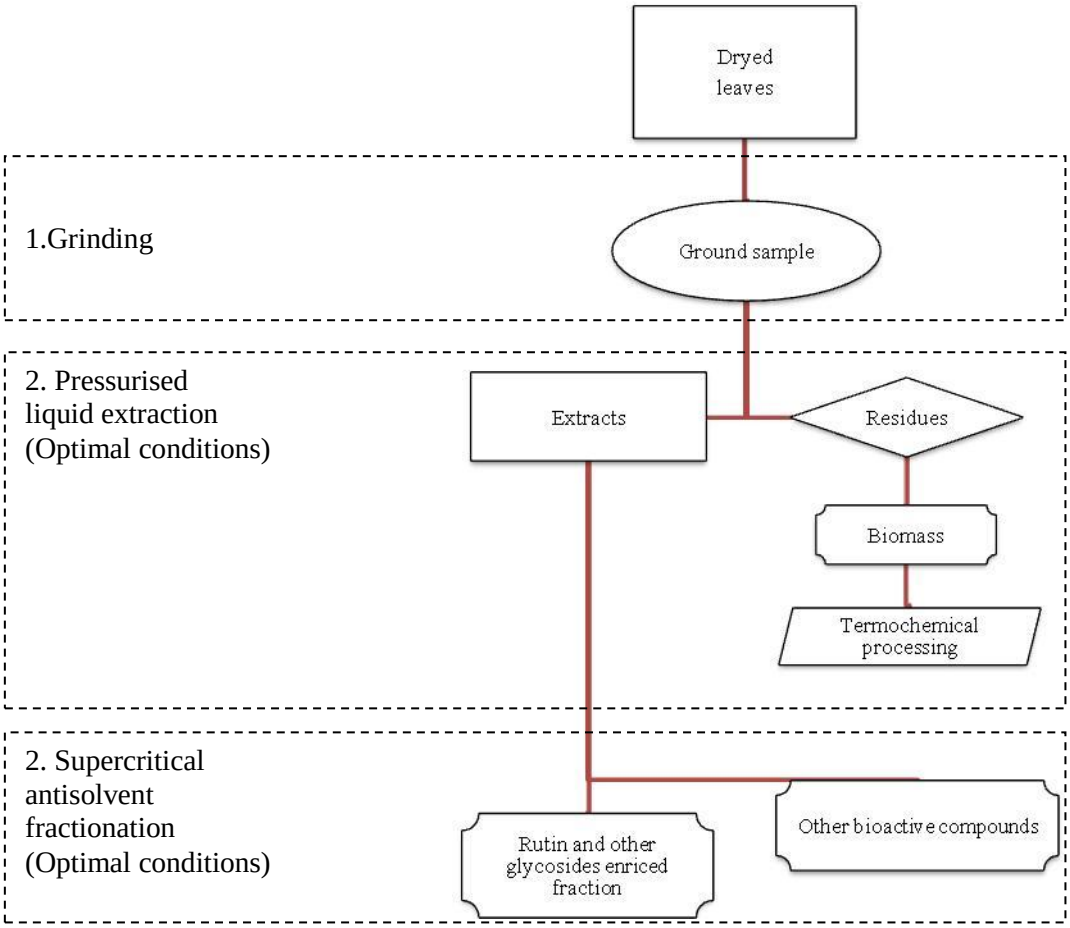


Fig. 4.22 Scheme of bio-refinery of amaranth leaves

V. CONCLUSIONS

1. Pretreated amaranth seeds were separated into optimal size, composition and functional properties fractions: high amount of oil and protein containing bran and starch containing perisperm.
2. Accelerated solvent extraction (ASE) may be applied for the isolation of lipids from amaranth; extraction rate is faster approximately 5 times as compared with the standard Soxhlet extraction procedure. The optimal conditions to obtain the highest total yield (13.7 g/100 g) were 17 min extraction time at the temperature of 90 °C. Under these conditions, the experimental value was similar to the predicted value. The highest oil yield was obtained from sample "Bio" grown in Bolivia 18.5 g/100 g and sample „Geltonukai" grown in Lithuania 16.8 g/100.
3. In the present study, the SCE-CO_2 conditions were optimised to obtain the highest extract yield at pressure 35.8 MPa, temperature 40 °C, CO_2 flow rate 2.9 SL/min, and extraction time 110 min. It was shown that depending on seed variety may be obtained up to 16.7 g/100 g ("Bio") of lipids from floured amaranth seed fraction (≤0.23 mm) at optimal conditions. Based on response plots, two extraction parameters, time and pressure were found to be the factors most significantly influencing extraction yield.
4. Amaranth seed oil with the highest content of squalene (14.88 g/100 g) obtained under 15 MPa pressure without ethanol and possessed the highest antioxidant activity. The highest amount of tocopherols (317.3 mg/kg) from amaranth seeds was achieved using 55MPa pressure and adding 5% of ethanol. Ethanol increased tocopherols yield and antioxidant activity up to 3 times, under higher extraction pressure (35-55 MPa). The fraction with high concentrations of squalene may be obtained at lower pressures (e.g. 15 MPa), while the fractions enriched with tocopherols and squalene may be obtained by gradual reduction of pressure in the separators until atmospheric conditions.
5. The extracts isolated from defatted leaves and flowers were remarkably stronger antioxidants using $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and TPC assays comparing to defatted stems and seeds. The extracts isolated with polar solvent methanol/water mixture were stronger antioxidants than the extracts obtained with acetone. Antioxidant power of the whole plant material evaluated by QUENCHER method was approximately 2 times higher than the integrated values obtained for the extracts; it suggests that considerable amount of antioxidatively active compounds remain in the plant material after extraction. A strong correlation between total polyphenols content and antioxidant activity measured by $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and ORAC was observed. On-line HPLC-$DPPH^{\bullet}$ assay of amaranth extracts showed that rutin was the main radical scavenger in amaranth.
6. Pressurised liquid extraction at subcritical conditions using water and ethanol mixture as a solvent was applied to maximized rutin content from amaranth leaves. At optimal conditions rutin recovery was 4 times higher comparing with conventional extraction. Using SAF method was able to separate water (high polarity)

and ethanol (medium polarity) soluble compounds. Rutin was recovered in rafinate with the highest concentration 22570 mg/kg, at 15 MPa and feed mixture flow rate 0.3 ml/min.

7. Amaranth anatomical parts examined in this study contain valuable functional constituents – squalene, tocopherols, rutin and others. Applying high pressure extraction and fractionation methods possible to obtain high-added value fractions which can be used in different areas, particularly in production of functional food and healthy supplements. Applied biorefinery conception increase the feasibility of practical realization of amaranth processing.

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VII. LIST OF PUBLICATIONS ON THE THEME OF THIS DISERTATION

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1. Paulius Kraujalis, Petras Rimantas Venskutonis. Optimisation of supercritical carbon dioxide extraction of amaranth seeds by response surface methodology and characterization of extracts isolated from different plant cultivars // The Journal of Supercritical Fluids. ISSN 0896-8446. 2013, vol. 73, p. 80-86 (ISI web of science, I.F. 3.138).
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3. Paulius Kraujalis, Petras Rimantas Venskutonis, Audrius Pukalskas, Rita Kazernavičiūtė. Accelerated solvent extraction of lipids from *Amaranthus* spp. seeds and characterization of their composition // LWT- Food Science and Technology. ISSN 0023-6438. 2013, vol. 54, no. 2, p. 528-534 (ISI web of science, I.F. 3.107).
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5. Petras R. Venskutonis, Paulius Kraujalis. Nutritional components of amaranth seeds and vegetables: A review on composition, properties and uses // Comprehensive Review in Food Science and Food Safety. ISSN 1541-4337. 2013, vol. 12, no. 4, p. 381-412 (ISI web of science, I.F. 5.053).

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2. Paulius Kraujalis, Petras Rimantas Venskutonis. Optimization of Supercritical Carbon Dioxide Extraction Parameters for Amaranth Seeds. // 2012 IFT Annual Meeting and Food Expo Technical program : Las Vegas, Nevada, U.S.A., June 15-28, 2012 : book of abstracts 2012. ISSN 1082-1236 p. 68.
3. Kraujalis, Paulius, Venskutonis, Petras Rimantas, Antioxidant activity of amaranth seed fractions isolated by different solvents. // Foodbalt-2012 : Innovate and healthy food for consumers : 7th Baltic Conference on Food Science and Technology, Kaunas, May 17-18 : conference program and abstracts / Kaunas University of

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4. Kraujalis, Paulius, Venskutonis, Petras Rimantas, Optimization of supercritical carbon dioxide extraction parameters for Amaranth seeds. // 2012 IFT Annual Meeting and Food Expo Technical program : Las Vegas, Nevada, U.S.A., June 15-28, 2012 : book of abstracts Chicago : Institute of food technologists. ISSN 1082-1236. 2012, p. 68.
5. Kraujalis, Paulius, Venskutonis, Petras Rimantas, Optimisation of isolation of amaranth lipophilic constituents by pressurized solvent and supercritical carbon dioxide extraction. // ISSF 2012 [elektroninis išteklius]: 10th International Symposium on Supercritical Fluids, May 13-16, 2012, San Francisco, Ca, USA : final program. San Francisco : [s.n.], 2012. p. [1].
6. Venskutonis, Petras Rimantas; Kraujalis, Paulius. Optimisation of supercritical carbon dioxide extraction and fractionation of amaranth (Amaranthus sp.) seeds // Proceedings of the 10th Conference on Supercritical Fluids and Their Applications, April 29-May 6, 2013, Napoli, Italy / Ed. E. Reverchon and I. De Marco. Fisciano : University of Salerno, 2013. ISBN 8878970611. p. 33-38. [0,500]. [Indėlis grupėje: 0,833].
7. Kraujalis, Paulius, Venskutonis, Petras Rimantas, Antioxidative properties of rich in squalene Amaranth seed oil extracted by supercritical carbon dioxide. // Foodbalt - 2013 : 8th Baltic conference on food science and technology "Food, health and well-being", May 23-24, 2013, Tallinn, Estonia : conference programm and abstracts / Tallinn University of Technology. Tallinnn : Tallinn University of Technology, 2013. ISBN 9789949430635. p. 37.
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