Statement of the supervisor

I, **Dr. Erika Vági**, supervisor, hereby declare that the thesis written by **Sergio Alonso Muñoz**, (Neptune code: BEWOK8), titled: **Optimization of extraction of bioactives from sea buckthorn (Hippophae rhamnoides L.) woody waste** is his own writing prepared under my supervision. I also declare that the thesis meets the formal and professional requirements of the Budapest University of Technology and Economics and those of the Faculty of Chemical Technology and Biotechnology, thus I support its submission.

06/6/2019
Budapest

..........................................................

(supervisor)

Statement of the student

I, **Sergio Alonso Muñoz** (Neptun code: BEWOK8) as author of the thesis hereby declare that my thesis titled: **Optimization of extraction of bioactives from sea buckthorn (Hippophae rhamnoides L.) woody waste**, is my original writing and I have not plagiarised any other work. All third party materials including published and unpublished sources were referenced.

I acknowledge that the intellectual property rights of the methods used and the results of any research or development described in the thesis belong to the participating researchers and institutions/companies, thus their utilization or publication must not be initiated before the approval of all parties.

I also declare that during the preparation and writing the thesis I did not mislead my supervisor(s) and thesis advisor.

06/6/2019
Budapest

..........................................................

(student)
Acknowledgement

I want to thank my parents first of all for all the unconditional support they have given me, without them none of this would have been possible.

I want to thank the universities from both Budapest and Valladolid for their great work.

And especially I want to thank Dr. Erika Vági, thank you for all the effort and dedication you have put in me.
BSc. Thesis

Optimization of extraction of bioactives from sea buckthorn (*Hippophae rhamnoides* L.) woody waste

Written by: Sergio Alonso Muñoz
BSc. in Chemical Engineering

Supervisor: Dr. Erika Vági
assistant professor

2019.
# Table of contents

1. Introduction........................................................................................................... 6
2. Objectives............................................................................................................... 7
3. Theoretical background............................................................................................ 8
   3.1 Sea buckthorn (*Hippophae rhamnoides* L.) ....................................................... 8
      3.1.1 Uses and benefits of sea buckthorn ............................................................. 9
      3.1.2 Cosmetical usage......................................................................................... 9
      3.1.3 Health benefits........................................................................................... 11
   3.2 Extraction methods ............................................................................................ 12
      3.2.1 Stirred tank extraction ............................................................................. 12
      3.2.2 Soxhlet extraction ................................................................................... 13
      3.2.3 Extraction solvents .................................................................................. 15
   3.3 Antioxidants ....................................................................................................... 15
      3.3.1 Determination of antioxidant activity by means of DPPH (2,2-diphenyl-1-picrylhydrazyl) ................................................................. 17
      3.3.2 Study of the oxidative capacity of oils by Rancimat method ..................... 18
4. Materials and methods .......................................................................................... 20
   4.1 Material and chemicals .................................................................................... 20
   4.2 Methods .............................................................................................................. 21
      4.2.1 Determination of moisture content ........................................................... 21
      4.2.2 Particle size distribution ........................................................................... 22
      4.2.3 Stirred tank extraction .............................................................................. 23
      4.2.4 Soxhlet extraction .................................................................................... 29
      4.2.5 Antioxidant activity of samples ................................................................ 30
      4.2.5.1 Antioxidant activity measured by DPPH method ................................. 30
      4.2.5.2 Study of protective effect of the extract by Rancimat method ............. 33
5. Results & discussion ............................................................................................... 36
   5.1 Characterization of sea buckthorn stalks ............................................................ 36
   5.2 Results of extraction .......................................................................................... 37
      5.2.1 Effect of temperature on the extraction yield .......................................... 40
      5.2.2 Effect of water content of EtOH-water extraction solution .................... 42
      5.2.3 Repeatability of experiments ................................................................... 44
      5.2.4 Re-extraction of extracted residue ........................................................... 44
   5.3 Antioxidant activity of sea buckthorn extracts ................................................. 44
      5.3.1 Antioxidant activity of sea buckthorn extracts by DPPH method ........... 44
5.3.2. Protective effect of sea buckthorn extracts evaluated by Rancimat method ........47

6. Conclusion .................................................................................................................50

7. References ...............................................................................................................52
1. Introduction

Nowadays products from sea buckthorn can be easily found as the berry of this easily grown, widely distributed shrub is very important source of vitamins, minerals, natural antioxidants, amino acids, fatty acids and other bioactive substances. As a natural medicine it has potential for arthritis, gastrointestinal ulcers, gout and skin rashes and irritations [1-4].

The fruits of sea buckthorn can also be used in the day to day to make jams, juices or oils. The fruit, leaves and seeds of sea buckthorn has been used for treatment of skin disorders, stomach malfunctioning, thrombosis, hepatic injuries, in Asian traditional medicine. The leaves of sea buckthorn are rich in phenolic compounds and possess strong antioxidant activity [3-6].

The stalk or stem of sea buckthorn is the part of the plant that is going to be investigated in this project. The stalks until now have been considered as a waste.

The main objective of the research is studying the antioxidant activity of the extracts of stem and branches of sea buckthorn, using different extraction methods such as stirred tank extraction and Soxhlet extraction in laboratory scales with different solvents such as pure ethanol, water, ethanol-water solutions and n-pentane. The optimization of extraction conditions, such as water content in the ethanol-water solutions, the extraction temperature is the main focus of this work for producing an extract which has strong antioxidant activity.

The antioxidant activity of extracts is analysed by the DPPH method using spectrophotometer, in which the hydrogen-donor activity of extracts can be revealed in the presence of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. On the other hand, the protecting effect of the extracts are also revealed by Rancimat fast oxidative measurement. Mixing together the extract with sunflower seed oil the extract might act as a protective agent which protect the oil from oxidation, therefore the shelf-life of oil can be increased. The analysis is carried out using Rancimat apparatus.

As a result of this work an optimized extraction method will be pointed out providing extract in high yield and with high antioxidant activity.
2. Objectives

1. Determination of the moisture content of samples.
2. Determination of the particle size distribution of the plant material.
3. Extraction of stalks using two different methods:
   3.1 Stirred tank extraction with different mixtures of ethanol and water and at different temperatures:
      - 96% Ethanol at 40°C, and 60°C
      - 70% Absolute ethanol 30% Distilled water (V/V) at 40°C, 60°C, 80°C
      - 50% Absolute ethanol 50% Distilled water (V/V) at 40°C, 60°C, 80°C
      - 30% Absolute ethanol 70% Distilled water (V/V) at 40°C, 60°C, 80°C
      - 100% Distilled water at 40°C, 60°C, 80°C temperatures.
   3.2 Soxhlet extraction with two different solvents:
      - 96% Ethanol
      - n-pentane
4. Determination of the antioxidant activity of samples with the DPPH method.
5. Analyse the oxidative capacity of the samples.
6. Compare the results and drawn conclusion.
3. Theoretical background

3.1 Sea buckthorn (*Hippophae rhamnoides* L.)

Sea buckthorn, also known *Hippophae rhamnoides* L., is a deciduous shrub within the genus *Hippophae*, belonging to the family *Elaeagnaceae*. It is native to a large area of Europe, Asia, Mongolia and Canada [1].

They reach heights of 0.5 to 6 m; rarely as high as 18 m in Central Asia and are generally found in dry, sandy areas. They are tolerant to salt in the air and soil but have full sun exposure requirements for full development and do not tolerate shade conditions near large trees.

The name of the plant comes precisely from the fact that they grow close to the sea and withstand extreme conditions from -40°C to 40°C degrees [2].

![Figure 1. Sea buckthorn distribution](image)

Sea buckthorn has fruits those are rich in essential nutrients such as fatty acids, vitamins -A, -C, -E and a wide variety of bioactive compounds such as antioxidants and phenolic compounds. The berries are typically used to make oils and to treat diseases of the digestive tract [3].

The leaves of sea buckthorn are rich in lipophilic compounds such as α-tocopherol, β-carotene and plastochromanol-8. The most common use is making tea which contains vitamins and minerals, antioxidants, amino acids and fatty acids. Tea is typically used to lower blood pressure and serum cholesterol, prevent and treat diseases of the blood vessel, and to increase immunity [4].

The stokes have common properties with berries and leaves but to a lesser extent. It is always taken as the waste of the plant. It was commonly used by the Greeks as food for horses [5].
3.1.1. Uses and benefits of sea buckthorn

There are seven varieties of sea buckthorn. The most common of which are the “Hippophae rhamnoides” (common yellowthorn), and the “Hippophae salicifolia” (willow-leaf cerval). The others are not so common as follows: “Hippophae gyantsensis”, “Hippophae litangensis” and “Hippophae tibetana”. Two other species: “Hippophae goniocarpa” and “Hippophae neurocarpa”, have been described in China, but are not widely accepted as distinct species [6].

Most of the world's sea buckthorn plantations are in China. The concentration of pro-vitamins A, B₂ and C is much higher than that of other fruits and vegetables such as carrots, tomatoes, oranges, etc [7].

<table>
<thead>
<tr>
<th>Species</th>
<th>Vitamin A</th>
<th>Vitamin B₁</th>
<th>Vitamin B₂</th>
<th>Vitamin C</th>
<th>Vitamin K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea buckthorn</td>
<td>11</td>
<td>0.04</td>
<td>0.56</td>
<td>300-1600</td>
<td>100-200</td>
</tr>
<tr>
<td>Kiwi</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100-470</td>
<td>-</td>
</tr>
<tr>
<td>Orange</td>
<td>0.55</td>
<td>0.08</td>
<td>0.03</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Tomato</td>
<td>0.31</td>
<td>0.03</td>
<td>0.02</td>
<td>11.8</td>
<td>-</td>
</tr>
<tr>
<td>Carrot</td>
<td>4</td>
<td>0.02</td>
<td>0.05</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 1 Comparison of the vitamin contents of sea buckthorn and others (mg/100g) [7].*

Presence of these antioxidant vitamins at high levels indicates their strong antioxidant property. The shrub serves as a warehouse for researchers in the field of biotechnology, nutraceuticals, pharmaceuticals, cosmetics and environmental sciences [7].

3.1.2. Cosmetical usage

The main use of sea buckthorn in this sector is the manufacture of an oil. The oil of sea buckthorn berry is very rich in omega 3, 6, 7 and 9 fatty acids (linolenic, linoleic, palmitoleic and oleic acids, respectively). It also contains a large amount of vitamin C (695mg/100 g), tocopherol or vitamin E (180 mg/100 g), folic acid (80 mg/100 g). It also provides carotenoids (lycopen, lutein, beta-carotene and zeaxanthin), flavonoids (quercetin, isorhamnetin-3-beta-D-glucoside, isorhamnetin, kaempherol, etc) [8].
This oil has excellent properties such as:

- A great anti-inflammatory. It is very useful for skin diseases such as psoriasis, eczema and dermatitis.

- Gastrointestinal and vaginal diseases.

- The oil of sea buckthorn hydrates the mucous membranes of the body being of great help in pathologies, like vaginal dryness or disease of the dry eye. It is very important for the skin due to its content in omega 7.

- Defences: Stimulates the immune system. It is cardio-protective therefore supports the health of the cardiovascular system and controls appetite so it is a great help to lose weight. There are several studies showing that sea buckthorn oil helps lower cholesterol and triglycerides.

- Help for the liver: It is also considered a liver tonic. Promotes detoxification of liver cells.

- Other uses such as: Obesity, stress, general wellbeing, oncological processes [8].

*Figure 2 Sea buckthorn oil*
3.1.3. Health benefits

Sea buckthorn (SBT) has been extensively studied for the treatment of stomach malfunction, thrombosis, hepatic lesion, tendon and ligament lesions and neoplasia.

Clinical trials in patients with ischemic heart disease have shown that total SBT flavonoids reduce the cholesterol level and improve heart function[9].

It is also established that sea buckthorn juice antioxidants reduce the risk factor for coronary heart disease in humans. Studies also demonstrated that SBT oil is effective in cancer therapy. The extracts from the leaves of sea buckthorn have been reported to have marked antibacterial, antiviral, and antitumor activities[9].

SBT extracts possess antibacterial activities and have also shown a protective effect against the toxic effect of mustard gas, a chemical warfare agent. Many of the claims associated with sea buckthorn relate to high nutritional value in terms of vitamins, organic acids, flavonoids, macro and micronutrients elements[9].

Figure 3 Sea buckthorn stalks tea
3.2. Extraction methods

Solid-liquid extraction is a basic or unitary operation that separate the desired solute or remove an undesirable solute component from the solid phase, while the solid is contacted with the liquid phase. The two phases are in intimate contact and the solutes or solute can diffuse from the solid to the liquid phase, this is the main objective of solid-liquid extraction [10].

In a solid-liquid extraction process the operations involved are:

- Phase change of the solute. This stage is considered practically instantaneous.
- Diffusion of the solute through the solvent contained in the pores of the inert solid.
- Transfer of the solute from the proximal areas of the S/L interface to bulk of the solvent.

Consequently, the most important factors influencing the extraction speed are:

a) Size of solid particles. Evidently, the smaller they are, the larger the interfacial surface and shorter the length of the pores. Therefore, the greater the transfer speed. However excessively small sizes can make the particles clump together, making extraction difficult.

b) Type of solvent. The solvent should be as selective as possible and it is recommended that has low viscosity.

c) Temperature. An increase in temperature favours solubility and increases the extraction speed.

d) Agitation of the solvent-solute. It favours the transfer by increase of mass transfer coefficients in the solid-liquid interface. It also prevents sedimentation and caking of solid particles[11].

Two types of solid-liquid extractions will be investigated:

3.2.1. Stirred tank extraction

This is a discontinuous agitated tank reactor composed of an agitation unit that goes into the round bottom flask or into the tank reactor and a heated unit or jacket that will provide the mixture the necessary temperature to produce the appropriate solid liquid separation.

The reactor must be maintained at constant temperature and stirring rate parameters.

Inside the round bottom flask or reactor, the solvent is mixed with the solute.
The unit is equipped with a thermometer to keep the temperature constant. It is fixed at all times by means of metal brackets that fix the structure.

The main applications of the batch reactor are the fine chemical industry, discontinuous polymerization, organic chlorination of small intermediate products or large tonnage petrochemical basic products[12].

### 3.2.2 Soxhlet extraction

The Soxhlet extraction chamber was invented by Franz von Soxhlet in 1879. At first it was created to determine the fat in food[13].

Soxhlet extraction has 4 distinct stages:

1. Placing the solvent in a volumetric round bottom flask.
2. Boiling of the solvent that evaporates to a reflux condenser.
3. The condensate falls on top of the porous cartridge containing the sample inside.
4. Collecting of the solvent level to a point where reflux occurs that returns the solvent with the extracted material back into the round bottom flask.

The process is repeated as many times as necessary until the sample is exhausted. The extract is accumulated in the round bottom flask soluble in solvent.

The sample is deposited in some cartridges. They are porous and semi-cylindrical and are covered with cotton to avoid that it rushes out or between solvents inside.

The extraction is run in reflux for several days until the solute has been completely extracted.

This device has an advantage that sample is always being extracted with the pure solvent as it evaporates at its boiling point and came into contact with the sample material in the extraction chamber.
As we can see in *Figure 4*, the Soxhlet equipment is formed by:

A. Round bottom flask with the solvent  
B. Distillation path  
C. Condenser  
D. Extraction chamber with porous thimble  
E. Siphon
3.2.3. Extraction solvents

Polarity is a widely used concept when explaining the separation of certain elements. It can be said that it has a certain effect on certain substances in which depending on whether they are polar or not they will have a greater capacity to dissolve solutes whose polarity is similar to them.

The ethanol, water and their mixtures used for the extraction of antioxidants are polar solvents, as is well known by the theory a polar solute tends to dissolve in a polar. On the other hand, n-pentane was also used, which is a highly non-polar solvent resulting extract with different character and polarity.

3.3. Antioxidants

An antioxidant is defined as any molecule capable of preventing or slowing the oxidation (loss of one or more electrons) of other molecules, usually biological substrates such as lipids, proteins or nucleic acids. Oxidation of such substrates may be initiated by two types of reactive species: free radicals, and those species that are not free radicals, but are sufficiently reactive to induce oxidation of such substrates.

Antioxidants consumed through food have an important potential to reduce the development of those diseases that currently affect the world’s population (cardiovascular diseases, tumours and neuro-degenerative)[14].

Antioxidants are divided into two large groups:

- **Endogenous**: those that are biosynthesized by the body:
  a) Enzymes: these are the proteins in charge of maintaining the levels of acceptable free radicals in our cells.
  b) Superoxide coenzyme: they regulate the arrival of oxygen to the cells, thus avoiding excessive oxidation of the cells.
  c) Catalase: captures free radicals and converts them into water and oxygen, thus benefiting the cells.
  d) Uric acid: has neuroprotective action helping to protect the nervous system.
- **Exogenous**: those obtained through a diet which are non-enzymatic such as:

  a) Vitamin C: facilitates electrons to free radicals helping to reduce the oxidation of cells.
  b) Alpha-lipoic acid (ALA): it is responsible for reducing inflammations is easier to transport to the brain than other antioxidants and facilitates the regeneration of other antioxidants such as vitamin A and C.
  c) Glutathione also known as master antioxidant is found in all cells of the body.
  d) Polyphenols: A compound found in fruits and plants. Its antioxidant action is to react to various heavy metals such as iron or copper and thus prevent the formation of free radicals in the molecule[15]

- **Synthetic antioxidants**

  Synthetic antioxidants develop from the need for more effective protection than natural antioxidants, and offer a more economical option relative to natural antioxidants.

  Butil hydroxi-toluene (BHT) and butil hydroxi-anisol (BHA) are the most important synthetic antioxidants.

  Its main functions are:

  1. The first function of synthetic compounds is to conserve the organoleptic properties and to preserve the nutritional quality of the food.
  2. Prolong the shelf life of industrialized foods.
  3. Once the compound has been intentionally introduced into the food, its function as an antioxidant does not stop the formation of radicals that are generated in oxidation[16].
3.3.1. Determination of antioxidant activity by means of DPPH (2,2-diphenyl-1-picrylhydrazyl)

In order to know the activity of antioxidants presented in food, herbs, plants the most commonly used method is that of DPPH, which is a free radical. Free radicals are molecules that are characterized by having one or more electrons in their outermost layer condition that makes them highly reactive.

![Figure 5 DPPH free radical](image)

When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present) [17].

![Figure 6 Diphenyl-picryl-hydrazyl (nonradical)](image)

Representing the DPPH radical by $Z'$ and the donor molecule by $AH$. The primary reaction is:

$$Z' + AH = ZH + A$$
This disappeared electron causes a colour change of DPPH from its violet colour turns to pale yellow as it reacts with an antioxidant species being measured with spectrophotometer at a wavelength of 517 nm.

The percentage of DPPH free radical uptake is determined by absorbance difference.

It is a reaction with a kinetic of pseudo-primer order that can continue measuring the decrease in absorbance over time.

The results are expressed as the value of IC$_{50}$ which is defined as the concentration of the test sample that produces an inhibition of 50 % of the free radical of DPPH. It is therefore said that the IC$_{50}$ value depends on the nature of the antioxidant compound and the concentration of DPPH [17].

### 3.3.2. Study of the oxidative capacity of oils by Rancimat method

The Rancimat is a method of measurement of the oxidative stability of oils and fats under accelerated conditions. This method is based on the induction of oxidation of the sample by exposure to high temperature and airflow.

The equipment (Figure 7) has two aluminium heating blocks with electrical heating which can operate at the same or different temperatures. It has a capacity for 8 samples (4 positions per block). It admits a temperature range: from 50ºC to 220ºC in divisions of 1ºC. It has an air flow capacity of 7-25 L/h[18].

The volatile oxidation products are transferred to the measuring vessel by the air stream and absorbed there in the measuring solution (distilled water). When the conductivity of this measuring solution is recorded continuously an oxidation curve is obtained whose point of inflection is known as the induction time[19].
The main applications of the Rancimat method are:

- Oxidation stability of fats and oils of animal and vegetable origin.
- Examination of the effectiveness of antioxidants.
- Oxidation stability of foods and cosmetics containing fats and oils[20]

Figure 7. 743 Rancimat equipment [19]
4. Materials and methods

4.1. Material and chemicals

The material used for the extraction was sea buckthorn stalks given by a local bio food processing company, Bio-drog-Berta Ltd., (Kalocsa, Hungary).

The material contains ground particles of stalks and stems of sea buckthorn plant in the form of a small splinter it has brown colour and the smell is similar to natural horse food.

![Grinded sea buckthorn plant](image)

*Figure 8. Grinded sea buckthorn plant*

The following chemical were used during the experiments:

- Ethanol (C₂H₆O) supplied by Molar Chemicals Kft. Purity: 96%
- Acetone (C₃H₆O) supplied by Molar Chemicals Kft. Purity: 99.95%
- Absolut ethanol (C₂H₆O) supplied by Molar Chemicals Kft. Purity: 99.98%
- Distilled water: from laboratory.
- n-Pentane (C₅H₁₂) supplied by Molar Chemicals Kft. Purity: 98.03%
- DPPH or 2,2-diphenyl-1-picrylhydracryl, free radical (C₁₈H₁₂N₅O₆) supplied by Sigma-Aldrich Co.
- Methanol (CH₄O) supplied by Molar Chemicals Kft. Purity: 99.5%
- Sunflower oil (Bunge Ltd, Hungary)
- 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) supplied by Fluka Chemie GmbH. Purity 99%.
- terc-butil-4-hidroxianisol (BHA) supplied by Fluka chemie GmbH. Purity 98%.
4.2. Methods

4.2.1. Determination of moisture content

First of all, the moisture content of sea buckthorn was measured. For this 3 parallel of measurements were carried out in order to obtain the highest possible accuracy.

Each sample was approximately 10 g of the material. The glass without material was weighed first then the 10 g of material was introduced and weighed. The samples were kept in the oven at 105°C for minimum one day in order to dry the material completely (until mass constancy).

After complete dryness, the sample was taken out of the oven and weighed again. The moisture content was calculated using the following formula:

\[
DC (%) = \frac{M_2}{M_1} \times 100
\]

*Equation 2. Calculation of dry content*

The average of the three samples was taken.

Where:

- DC is the dry content of plant material (%)
- \( M_1 \) is the initial weigh of plant material before been dried in the oven
- \( M_2 \) is the weight of plant material after been dried in the oven

The moisture content can be calculated as:

\[
MC = 100-DC (%)
\]

*Equation 3. Calculation of moisture content*
4.2.2. Particle size distribution

During this measurement, the particle size distribution was determined. A Retsch vibratory sieve shaker (Figure 9) was used with different sieve plate sizes (1.6; 1.4; 1; 0.8; 0.63; 0.4; <0.5mm).

- The empty plates were weighted before sieving.
- Sieve plates were assembled into a column with an empty tray at the bottom and with the largest diameter sieve plate at the top.
- Around 85.00 g of plant material was weighted on the top of the sieve plates.
- It was set for 20 minutes at 30 Hz amplitude shaking.
- After that, each sieve plate was weighted back with the residue.
- The percentage of the residue particles were calculated.

The experiment is repeated 3 times in order to obtain the highest possible accuracy.

The percentage of the different sizes was calculated by means of the formula:

\[
\% = \frac{M}{M_t} \times 100
\]

Equation 4. Calculation of percentage of different sizes on each sieve plates

where \( M_t \) (g) is the total mass of material introduced into the column and \( M \) (g) is the mass of material obtained from each plate.

Figure 9.3 Retsch vibratory sieve shaker
4.2.3. Stirred tank extraction

This was the most used method in the experiments for the extraction of antioxidants from sea buckthorn stalks. The results will be compared with those of the Soxhlet method in order to determine which of the two methods is the most efficient.

The following experimental design was planned to investigate the effect of temperature and H₂O content in EtOH on the yield of extraction.

First, 50 grams of the material was weighed and placed in a flask with a round bottom flask. Then the solvent was measured with volumetric cylinder. Ethanol-water solutions were used as extraction solvent. The proportion of ethanol was reduced as the experiments progress in such a way that one starts with 96% ethanol, 70-30% (V/V) ethanol-water, 50-50% (V/V) ethanol-water, 30-70% (V/V) ethanol-water and finally 100% distilled water.

The feed solvent ratio was kept unchanged during the experiments, 50 grams of plant material in 350 ml of extraction solvent (which is 1:7 m/V ratio).

The flask with the solvent and the material was placed on a support with a heating plate and a stirrer (Figure 10).

The temperatures for each proportion of solvent was varied starting with 40 °C then 60 °C and finally 80°C except for 96% ethanol because at 80°C the solvent would evaporate.

Agitation was kept constant in all experiments at a speed between 440 and 460 rpm approximately so that the sample was in constant motion and uniformly mixed.
After three hours of heating and agitation the mixture was filtered to separate the SBT content from the solution with the extract. For this purpose, vacuum filtration method was used by means of a Buchner funnel and a suction bottle.

The material of interest stayed in the flask that will later be analysed to obtain the activity of the antioxidants.

The dried extract in the flask was weighted. The extract was taken out from the flask and collected in a sample bottle.

The extraction yield was calculated as follows:

\[
Y_{\text{ext.}} = \frac{m_{\text{extract}}}{m_{\text{dry material}}} \times 100 \quad \text{[g/100 g dry material]}
\]

Equation 5. Calculation of extraction yield

The evaporation can be done in two different ways: by vacuum rotary evaporator (Heildolp, Germany, Figure 11 or with an automated solvent evaporation system (Biotage TurboVap) LV, Germany). The method is chosen according to the proportion of water in the solution since the water has a higher boiling point and will need a higher vacuum, temperature and longer time to evaporate.
Vacuum rotary evaporator

The theoretical principle of the rotary evaporator is to decrease the pressure in the mixture in order to lower the boiling point of the solvent to be evaporated.

The fact that it is a rotary evaporator has advantages over non-rotary evaporators:
- The centrifugal and the friction force between the wall of the rotating flask and the liquid sample result in the formation of a thin film of hot solvent that spreads over a large surface and ends with the extract adhered to the wall.
- The forces generated by the rotation suppress the blows, along with the above characteristics make the evaporation go smoothly and quickly.

The main disadvantage of rotary evaporators is that mixtures such as ethanol and water lead to the formation of bubbles and foams that could hinder evaporation, for this must be controlled and high bath temperature and pressure to which the mixture is subjected[21].

Therefore, for certain experiments, the Biotage TurboVap was used.

![Vacuum rotary evaporator](image)

*Figure 11. Vacuum rotary evaporator*
Biotage TurboVap

The glass used to contain the solution that evaporates must first be weighed empty and then with the extract in order to know the solid content that remains for later analysis and thus make a comparison between the different tests.

Large volume glass tubes (250 ml each) were used to evaporate the solvent above the extract. After weighing the glass tubes, filtrate was added up to half of volume of total volume of glass tubes.

The glass tubes were placed into a rack then into the water bath (with adjustable temperature) of the equipment.

The apparatus was closed; the air flow was adjusted as required. When the level of solvent was higher, the air flow was kept at lower range (1-2 L/min); then as the sample dried the air flow was increased up to 4 L/min.

The air was supplied though nozzles onto the top of samples; while the airflow was provided by a compressor.

Once the samples completely dried, the tubes were weighted back, the extract was collected and stored in sample bottle in the fridge for further analysis.

Figure 12 Biotage TurboVap evaporating system
The residue obtained in the form of sea buckthorn splinters and was used to calculate the mass balance.

\[ m_{\text{initial plant material}}(g) \times \frac{DC}{100} = m_{\text{extract}}(g) + m_{\text{dried residue}}(g) + \text{loss}(g) \]

And then the mass balance error was calculated as follows:

\[ \text{Mass balance error} = \frac{m_{\text{loss}}}{m_{\text{initial}}} \times 100 \quad (\%) \]

- Re-extraction of extracted plant material

The stirred tank extraction with 96% EtOH was carried on the residue plant material after the 3h extraction at 40°C and 60°C was extracted with the same solvent at 40°C and 60°C for another 3h.

The solvent was filtrated and evaporated, while the re-extracted plant material was further extracted for another 1h with 96% EtOH at 40°C and 60°C.
The following steps were followed:

Residue I
- Stirred tank 96% EtOH at 40°C for 3h

Residue II
- Stirred tank 96% EtOH at 40°C for 1h

Residue III
- Dried

The yield of these step-wise extraction was calculated and the extracts were kept separately for further analysis.

- Repetition of stirred tank extraction

In the middle point (at 60°C with 50% EtOH-water solution) of experimental design three parallel measurements were carried out to obtain the accuracy of this experiment.
### 4.2.4. Soxhlet extraction

In this procedure the solid sample is placed in a porous material cartridge that occupies approximately ¾ of the cartridge and covered with cotton-wool to prevent the solvent from being introduced into the interior of the cartridge and mixed with the material.

The extractant solvent in the flask is heated by means of a silicon oil. Its vapours are condensed and fall, drop by drop, on the cartridge containing the sample, extracting the soluble analytes. When the level of the condensed solvent in the chamber reaches the top of the side siphon, the solvent, with the dissolved analytes, rises up the siphon and returns to the boiling flask.

The solvent is left to reflux for 1-3 days, depending on the raw material and the solvent used.

This process is repeated until the extraction of the analytes from the sample is the sample was fully exhausted; the solvent above it was clear and transparent.

![Figure 4 Soxhlet equipment](image_url)
Two different solvents were used in the experiments: *n*-pentane and 96% EtOH.

The sample is ready when the reflux liquid becomes a transparent colour (its normal colour) that means that the hot solvent had extracted all the soluble analytes that were in the sample.

This method is not equipped with agitation like the stirred tank and also needs a longer time than that however the extraction temperature will be higher than in the stirred tank.

Once the process is finished the ethanol and *n*-pentane were evaporated in order to get the solid extract. The rotary evaporator was used in the case of the *n*-pentane and 96% ethanol as they were easy to evaporate.

The extract then was weighed back, taken out from the flask and stored in small sample containers until further analysis.

### 4.2.5. Antioxidant activity of samples

The antioxidant activity of extracts from sea buckthorn stalks were measured in two different ways. Firstly, the scavenging activity of sea buckthorn extract was measured in the presence of DPPH free radical using spectrophotometer. Secondly the extracts were added to vegetable (sunflower seed) oil to measure whether the extracts had any protective activity towards the applied oil in a fast induced oxidative environmental.

In the first assay the bioactive compounds of extracts act in polaric phase (in methanol), while with the oil-assay these or other compounds may act in oily, highly apolaric phase.

#### 4.2.5.1 Antioxidant activity measured by DPPH method

Firstly, the DPPH solution was needed to be prepared. 20 mg of DPPH (2,2-diphenyl-1-picrylhydrazyl) was weighted out on an analytical scale and solved in 50 ml of methanol placing it in a brown sample bottle for light protection as the solution deteriorates with light.

This concentrated solution of DPPH needed to be further diluted to a solution with an absorbance between 0.7 and 0.9 (at 517 nm wavelength) and this was achieved by diluting the concentrated solution of DPPH with methanol.

To prepare the sample for analysis: 10 mg of the sample was weighed on a precision electronic laboratory scale and solved in 20 ml of methanol (0.4 mg/ml). It was stored in dark sample bottles.
Once the solution had been prepared it was placed in an ultrasonic mixer in order to obtain a homogeneous mixture and fully dissolved sample.

The spectrometer was switched on and configured so that the wavelength was set at 517 nm and only the UV light was switched on in order to measure the absorbance of the sample at that wavelength.

Firstly, 100% methanol in a cuvette was used as blanking the spectrophotometer.

2.5 ml of DPPH solution was pipetted into each cuvette which shows its characteristic purple colour, and 100 µl, 200µl, 300µl, 400µl and 500µl of the sample were pipetted to this solution. The cuvettes were mixed with vortex mixer; covered with foil and kept in dark for 30 minutes.

After half an hour the cuvettes were taken out of the dark cabinet and the absorbance was measured at 517 nm.

Three parallel measurements for each extracts were carried out. For comparison the antioxidant activity of synthetic antioxidants (BHT and BHA) were also measured.

The percentage of inhibition of DPPH radical was calculated using the following formula:

\[
\text{Inhibition(\%)} = \frac{A_0 - A_1}{A_0} \times 100
\]

Where:

A0, is the absorbance of the diluted solution without extract sample (control DPPH solution).

A1, is the absorbance of a cuvette with extract solution.

Then the calculated inhibition was plotted against the concentration and the IC50 value could obtained from the graph (as seen on Figure 15).
The change of colour from purple to yellow of DPPH indicates that the extract has antioxidant activity and it depends on the applied concentration of it.
4.2.5.2. Study of protective effect of the extract by Rancimat method

The main objective of this experiment was to compare the oxidative activities of vegetable oil samples with and without added sea buckthorn extracts obtained with Soxhlet and stirred tank extraction. In the Rancimat method the auto-oxidation of oils and fats are fasten by strong stream of air at high temperature. Volatile oxidation products cause changes in conductivity, which is monitored during the experiment.

Therefore, the inflection point of oxidation curve can be measured and recorded as an induction time, that provides good characteristic value for the oxidation stability. These recorded induction times of control sunflower oil and of the sunflower oil containing the sea buckthorns extracts can be compared. If the oil samples which contain sea buckthorn extracts had longer induction time; it means that the added extract has protective effect which protects the oil from auto-oxidation. Then the extract might be used as natural preservative added to oily products. For qualification and quantification of these active compounds further studies are needed.

![Figure 7. Rancimat equipment](image-url)
For the measurement Rancimat 743 equipment was used (Figure 17) and firstly 8 test tubes were prepared. Two of them with the control sunflower oil (by weighing 3 grams of sunflower oil into a test tube), other test tubes contain the vegetable oil + sea buckthorn extract in 1% (w/w) concentration. Three parallel measurements were carried out for each extract.

Sea buckthorn extracts are mixed into the vegetable oil (sunflower oil) at 1% (w/w). If the extract contains protective compounds; the oxidative stability (induction time) will increase.

Once the samples were prepared in the test tubes then were assembled and placed into the previously lit Rancimat apparatus, which was preheated to a temperature of 120°C.
The measuring program was set at 120°C with 20 L/min air flow (default mode).

As soon as all reaction and measuring tubes were assembled; the program was started and it run automatically until the induction time of each samples was recorded.

After the recorded graph of conductivity versus time was obtained, copied and the recorded induction times were collected and evaluated further.

From the parallel measurements average induction time was calculated with standard deviation.

A characteristic graph in the presences of sunflower seed oil and oil with sea buckthorn extracts can be seen in Figure 21 and on Table 2. The measured conductivity is plotted against time and the observed induction times are spotted by triangles.

![Figure 21. Conductivity plotted against time with the recorded induction times of samples](image)

<table>
<thead>
<tr>
<th>ID 1</th>
<th>Determination</th>
<th>Temperature</th>
<th>Induction time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower oil</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>2.61 h</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>2.61 h</td>
</tr>
<tr>
<td>Oil+1% Seabuck Soxh</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>3.02 h</td>
</tr>
<tr>
<td>Oil+1% Seabuck Soxh</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>2.89 h</td>
</tr>
<tr>
<td>Oil+1% Seabuck Soxh</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>3.06 h</td>
</tr>
<tr>
<td>Oil+1% Seabuck ST1_1</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>3.10 h</td>
</tr>
<tr>
<td>Oil+1% Seabuck ST1_2</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>3.10 h</td>
</tr>
<tr>
<td>Oil+1% Seabuck ST1_3</td>
<td>04.04.2019</td>
<td>120°C</td>
<td>3.07 h</td>
</tr>
</tbody>
</table>

*Table 2. Recorded induction time by Rancimat measurement*
5. Results & discussion

5.1. Characterization of sea buckthorn stalks

As the stalks arrived to our laboratory in shredded form, and further grinding was not possible, firstly the particle size distribution of sea buckthorn stalks was evaluated. On Figure 22 the particle size distribution of the sample can be seen.

![Particle size distribution of sea buckthorn stalks](image)

Based on the results, it can be seen, that 30.7% of the particles of the sample possessed an average size of 1 mm, while the size of 80% of the particles was between 0.8 – 1.6 mm showing a very wide particle size distribution.

The moisture content of the shredded sea buckthorn was also measured. Three parallel measurements were carried out the data can be seen in Table 3.
The average dry content of sea buckthorn stalks was 92.94 ± 0.22 %, it contained around 7% moisture.

Once the dry content was obtained, it was applied to all calculation of yields of the extractions in the laboratory so the results are calculated back to dry plant material. Therefore, the obtained results can be easier comparable whether the sources of sea buckthorn are different for example by time of harvest, year, or due to geographic differences.

### 5.2. Results of extraction

The extraction of sea buckthorn stalks was carried out using traditional Soxhlet extraction with \(n\)-pentane and with 96% ethanol solvents. Also an experimental design was carried out to investigate the effect of extraction temperature and water content in the ethanol solution on the extraction yield. For this investigation experiments at different temperatures, such as 40 – 60 and 80°C and with different water content in EtOH (4, 30, 50, 70 and 100% water contents) were carried out in stirred tank apparatus at the same stirring speed, for 3 hours of extraction time and at the same solvent to feed ration (7:1 volume to mass ratio).

During the experiments the stirring speed and the temperature of water bath was kept constant to set at the required parameters.

The extraction yields were calculated as described in the Chapter 6.2.3. with Equation 5.

The extraction yields are summarized in Table 4.
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>T extraction (°C)</th>
<th>Solvent</th>
<th>Yield (g/ 100 g d.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST1</td>
<td>40</td>
<td>96% EtOH</td>
<td>1.47</td>
</tr>
<tr>
<td>ST2</td>
<td>60</td>
<td>96% EtOH</td>
<td>1.08</td>
</tr>
<tr>
<td>ST6</td>
<td>40</td>
<td>70% EtOH</td>
<td>2.47</td>
</tr>
<tr>
<td>ST4</td>
<td>60</td>
<td>70% EtOH</td>
<td>2.81</td>
</tr>
<tr>
<td>ST5</td>
<td>80</td>
<td>70% EtOH</td>
<td>3.50</td>
</tr>
<tr>
<td>ST7</td>
<td>40</td>
<td>50% EtOH</td>
<td>2.96</td>
</tr>
<tr>
<td>ST8</td>
<td>60</td>
<td>50% EtOH</td>
<td>3.15</td>
</tr>
<tr>
<td>ST16</td>
<td>60</td>
<td>50% EtOH</td>
<td>4.41</td>
</tr>
<tr>
<td>ST17</td>
<td>60</td>
<td>50% EtOH</td>
<td>4.55</td>
</tr>
<tr>
<td>ST9</td>
<td>80</td>
<td>50% EtOH</td>
<td>3.1</td>
</tr>
<tr>
<td>ST18</td>
<td>80</td>
<td>50% EtOH</td>
<td>4.19</td>
</tr>
<tr>
<td>ST10</td>
<td>40</td>
<td>30% EtOH</td>
<td>2.77</td>
</tr>
<tr>
<td>ST11</td>
<td>60</td>
<td>30% EtOH</td>
<td>3.20</td>
</tr>
<tr>
<td>ST12</td>
<td>80</td>
<td>30% EtOH</td>
<td>3.93</td>
</tr>
<tr>
<td>ST13</td>
<td>40</td>
<td>Distilled water</td>
<td>1.69</td>
</tr>
<tr>
<td>ST14</td>
<td>60</td>
<td>Distilled water</td>
<td>1.81</td>
</tr>
<tr>
<td>ST15</td>
<td>80</td>
<td>Distilled water</td>
<td>1.88</td>
</tr>
<tr>
<td>SOXHLET</td>
<td></td>
<td>96% EtOH</td>
<td>2.93</td>
</tr>
</tbody>
</table>

*Table 4. Extraction yields*
The results of Soxhlet extraction is summarized in Table 5.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>T extraction (°C)</th>
<th>Solvent</th>
<th>Yield (g/ 100 g d.m.)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOXHLET</td>
<td>boiling point</td>
<td>96% EtOH</td>
<td>2.93</td>
<td>0.12</td>
</tr>
<tr>
<td>SOXHLET</td>
<td>boiling point</td>
<td>n-pentane</td>
<td>0.32</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Comparing the results of Tables 4 and 5 we come to the conclusion that the extraction yield for the same solvent with 96% ethanol the yield was almost 3 times higher than that of achieved with the stirred tank apparatus. Perhaps the extraction time was too short with the stirred tank apparatus, and the Soxhlet extraction run much longer until the plant material was fully exhausted, which could have been achieved by a stirred tank extraction in 3 hours at lower temperature.
5.2.1. Effect of temperature on the extraction yield

First of all, the effect of extraction temperature on the extraction yield using different ethanol-water mixtures as extraction solvents are summarized.

Figure 24. Extraction yields with 70% EtOH

Figure 25. Extraction yields with 50% EtOH
As it can be seen from the graphs (Figure 24-27), as the extraction temperature increases the extraction yield increases with them whether 70% ethanol or distilled water was used as extraction solvents. The yields were the smallest comparing them to the other yields with ethanol in the extraction solvents, as the yields increased from 1.7 to 1.9 % (g/ 100 g dry material) by increasing the temperature. Comparing all the other results there was 1.4-fold higher increased with increasing the extraction temperature from 40°C to 80°C. Using 30% EtOH the yields increased from 2.8 – 3.9 %, using 50% ethanol solvent the yields increased from 3.0-4.6%, and applying 70% EtOH the extraction yields increased from 2.5-3.5%, which is all 1.4-fold increase. On the other hand, this did not happen with the extraction of 50% in which the extraction yield was the highest at temperature of 60°C (4.04±0.77 %), but as the standard deviation of three repeated measurements show a larger deviation, the difference between the extraction yields obtained with 50% ethanol are not significant. Comparing the results, the highest extraction yield was achieved with 50% ethanol-water solution at 60°C.
5.2.2. Effect of water content of EtOH-water extraction solution

The effect of water content from 4% to 100% in the ethanol-water extraction solution on the extraction yields are also investigated and summarized below.

![Figure 28. Extraction yields of stirred tank extraction at 40°C using different ethanol-water extracting solutions](image1)

![Figure 29. Extraction yields of stirred tank extraction at 60°C using different ethanol-water extracting solutions](image2)
As can be seen, as the proportion of water in the mixture increases, the extraction yield increases until it reaches the maximum with 50% EtOH and at 60°C (yield: 4.04 ± 0.77 %) thereafter the extraction yield decreases as the solvent acquires a greater proportion of water. At low temperature the extraction yields increased from 1.5 to 3.0%, which is 2-fold increase between using 96% ethanol as solvent or 50% ethanol-water solvent mixture. Similar trend can be seen comparing the extraction yields at 60°C (Figure 29.). The lowest extraction yield obtained with 96% ethanol 1.1 % and it increased up to 4.04% obtained with 50% ethanol, which is a 4-fold increase, the most prominent among the obtained results. The extraction yields obtained at 80°C almost 2-fold increase was observed between the yields obtained with distilled water only (1.9%) and the yield obtained with 50% EtOH (average yield of two measurements: 3.65%).
5.2.3. Repeatability of experiments

Three experiments were carried out at the middle point of extraction design in order to obtain the highest possible accuracy carrying out the extraction in a stirred tank apparatus in laboratory scale. The average extraction yield was 4.04 ± 0.77. The standard deviation is not very wide.

5.2.4. Re-extraction of extracted residue

At 40°C with 96% ethanol further experiments were carried out according to the explanation in Chapter 6.2.3. The extracted residue from the 1st experiment (ST1) run with 96% EtOH at 40°C was dried in oven and re-extracted with 96% ethanol for another 3 hours at 60°C. The extraction yield was 1.0% at this stage. Then the extracted residue was separated, dried again in oven and further extracted with 96% ethanol at 60°C for 1 hour, achieving 0.7% yield. The cumulative extraction yield was 3.14% which is 2-fold higher than that of obtained after the first step.

<table>
<thead>
<tr>
<th></th>
<th>Yield</th>
<th>Re-Yield(+3h)</th>
<th>Re-Yield(+1h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST1</td>
<td>1.47</td>
<td>1.01</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 6. Extraction yields of re-extraction of residues with 96% EtOH at 60°C

5.3 Antioxidant activity of sea buckthorn extracts

5.3.1 Antioxidant activity of sea buckthorn extracts by DPPH method
In order to study the antioxidant activity of the sample, experiments were made with each of the samples following the steps described in Chapter 6.3.1.

The following table contains the results of IC$_{50}$ (μg/ml) values of each extracts.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>T extraction (°C)</th>
<th>Solvent</th>
<th>IC$_{50}$(μg/ml)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST1</td>
<td>40</td>
<td>96% EtOH</td>
<td>29.37</td>
<td>0.18</td>
</tr>
<tr>
<td>ST2</td>
<td>60</td>
<td>96% EtOH</td>
<td>33.18</td>
<td>1.02</td>
</tr>
<tr>
<td>ST6</td>
<td>40</td>
<td>70% EtOH</td>
<td>37.40</td>
<td>0.70</td>
</tr>
<tr>
<td>ST4</td>
<td>60</td>
<td>70% EtOH</td>
<td>25.35</td>
<td>4.06</td>
</tr>
<tr>
<td>ST5</td>
<td>80</td>
<td>70% EtOH</td>
<td>22.60</td>
<td>0.87</td>
</tr>
<tr>
<td>ST7</td>
<td>40</td>
<td>50% EtOH</td>
<td>23.47</td>
<td>2.51</td>
</tr>
<tr>
<td>ST8</td>
<td>60</td>
<td>50% EtOH</td>
<td>22.16</td>
<td>1.20</td>
</tr>
<tr>
<td>ST16</td>
<td>60</td>
<td>50% EtOH</td>
<td>21.41</td>
<td>0.87</td>
</tr>
<tr>
<td>ST9</td>
<td>80</td>
<td>50% EtOH</td>
<td>20.26</td>
<td>0.50</td>
</tr>
<tr>
<td>ST10</td>
<td>40</td>
<td>30% EtOH</td>
<td>27.00</td>
<td>1.50</td>
</tr>
<tr>
<td>ST11</td>
<td>60</td>
<td>30% EtOH</td>
<td>24.07</td>
<td>0.53</td>
</tr>
<tr>
<td>ST12</td>
<td>80</td>
<td>30% EtOH</td>
<td>24.02</td>
<td>1.33</td>
</tr>
<tr>
<td>ST13</td>
<td>40</td>
<td>Distilled water</td>
<td>39.35</td>
<td>9.04</td>
</tr>
<tr>
<td>ST14</td>
<td>60</td>
<td>Distilled water</td>
<td>48.65</td>
<td>4.2</td>
</tr>
<tr>
<td>ST15</td>
<td>80</td>
<td>Distilled water</td>
<td>34.72</td>
<td>7.2</td>
</tr>
<tr>
<td>SX-EtOH</td>
<td>96% EtOH</td>
<td></td>
<td>24.37</td>
<td>0.80</td>
</tr>
</tbody>
</table>

*Table 7. IC$_{50}$ values of sea buckthorn extracts obtained with DPPH method*

As is well known, the lower the IC$_{50}$ factor the greater the antioxidant activity of the sample, in our case, the strongest antioxidant activity was measured in the presence of the sample ST9 with an IC$_{50}$ of 20.26 ±0.50 μg/ml.
As it can be seen in Figure 32, the antioxidant activity of almost all extracts was similar and very strong, especially if we compare these IC₅₀ values to those obtained in the presence of synthetic antioxidants, BHT and BHA. The strongest antioxidant activity was measured in the presence of extract obtained at 80°C with 50% ethanol, but similar results were achieved in the presence of all other extracts obtained with 30%, 50% and 70% of ethanol-water solutions at different temperatures. The antioxidant activity was also strong (24.4 μg/ml) in the presence of extract obtained with Soxhlet extraction using 96% ethanol solely. It can be also stated that the molecules which are responsible for this activity are not heat-sensitive. Interestingly, the extracts obtained with pure water showed the least antioxidant activities with the biggest standard deviations.

The antioxidant activity of BHT and BHA was as follows:

<table>
<thead>
<tr>
<th></th>
<th>IC 50</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHT</td>
<td>34.57</td>
<td>3.69</td>
</tr>
<tr>
<td>BHA</td>
<td>7.96</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Table 8 Activity of synthetic antioxidants
A comparison of the antioxidant activity of sea buckthorn samples with synthetic antioxidants was made using the DPPH method.

As the results show, BHA shows the highest antioxidant activity (7.96 μg/ml), but the antioxidant activities of the sea buckthorn stalk extracts are also comparable with BHT antioxidant.

### 5.3.2. Protective effect of sea buckthorn extracts evaluated by Rancimat method

The protective effect of sea buckthorn extracts mixed with vegetable sunflower seed oil can be studied. The extracts were mixed into the oil in 1% (w/w) and the samples were oxidized by Rancimat method. If the induction time of oils containing sea buckthorn extracts increased the sample has protective effect and can prolong oxidation, therefore might be applied as natural preservative. The induction time of control, sunflower oil containing no extracts was also measured and the results can be compared. Table 8 shows the measured induction times (h) of sunflower oil + sunflower oil with sea buckthorn extracts. Three parallel measurements were carried out; therefore the standard deviations are also summarized.
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>T extraction (°C)</th>
<th>Solvent</th>
<th>Oxidation time (h)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>oil</td>
<td>-</td>
<td>-</td>
<td>2.54</td>
<td>0.15</td>
</tr>
<tr>
<td>ST1</td>
<td>40</td>
<td>96% EtOH</td>
<td>3.12</td>
<td>0.06</td>
</tr>
<tr>
<td>ST2</td>
<td>60</td>
<td>96% EtOH</td>
<td>2.63</td>
<td>0.14</td>
</tr>
<tr>
<td>ST6</td>
<td>40</td>
<td>70% EtOH</td>
<td>2.69</td>
<td>0.19</td>
</tr>
<tr>
<td>ST4</td>
<td>60</td>
<td>70% EtOH</td>
<td>2.40</td>
<td>0.08</td>
</tr>
<tr>
<td>ST5</td>
<td>80</td>
<td>70% EtOH</td>
<td>2.84</td>
<td>0.06</td>
</tr>
<tr>
<td>ST7</td>
<td>40</td>
<td>50% EtOH</td>
<td>2.67</td>
<td>0.17</td>
</tr>
<tr>
<td>ST8</td>
<td>60</td>
<td>50% EtOH</td>
<td>2.69</td>
<td>0.08</td>
</tr>
<tr>
<td>ST9</td>
<td>80</td>
<td>50% EtOH</td>
<td>2.67</td>
<td>0.04</td>
</tr>
<tr>
<td>ST10</td>
<td>40</td>
<td>30% EtOH</td>
<td>2.54</td>
<td>0.10</td>
</tr>
<tr>
<td>ST11</td>
<td>60</td>
<td>30% EtOH</td>
<td>2.55</td>
<td>0.15</td>
</tr>
<tr>
<td>ST12</td>
<td>80</td>
<td>30% EtOH</td>
<td>2.51</td>
<td>0.02</td>
</tr>
<tr>
<td>ST13</td>
<td>40</td>
<td>Distilled water</td>
<td>2.48</td>
<td>0.07</td>
</tr>
<tr>
<td>ST14</td>
<td>60</td>
<td>Distilled water</td>
<td>2.39</td>
<td>0.11</td>
</tr>
<tr>
<td>ST15</td>
<td>80</td>
<td>Distilled water</td>
<td>2.48</td>
<td>0.03</td>
</tr>
<tr>
<td>SOXHLET</td>
<td>at boiling point</td>
<td>96% EtOH</td>
<td>2.99</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Table 8. Induction time (h) of sea buckthorn extract mixed with vegetable oil*
At first glance, the results look very similar. The induction time of control sunflower seed oil was 2.5 ± 0.2 hours. When extract obtained with Soxhlet extraction using 96% ethanol added to the oil the induction time increased to 3.0± 0.1 h, which is a 1.2-fold increase compared it to the induction time of control oil. In the presence of extract obtained also with 96% ethanol but with stirred tank extraction at low temperature (40°C) the induction time increased to 3.1± 0.1 h, which is the highest and also shows a 1.2-fold increase. Based on these findings, sea buckthorn extracts obtained with 96% ethanol has a slight protective effect when mixed with vegetable oil in 1% concentration resulting a 1.2-fold increase in stability. It can be also stated that the compounds which are responsible for this activity are not heat-sensible but their solubilities are the highest in water-free extraction solvent.
6. Conclusion

The following conclusions have been drawn from the study carried out:

First of all, the size of the sea buckthorn stalks was evaluated. About 80% of the particles had a size between 0.8 mm and 1.6 mm. Being the size of 1mm the most common with 30.7%.

The dry content of sea buckthorn stalks was 92.94 ± 0.22 %, it contained around 7% moisture.

With the two methods that were used for the extraction of antioxidants from the plant sea buckthorn can be said to be almost 3 times higher extraction yield obtained by the Soxhlet method (2.93 ± 0.12) than the stirred tank at 96% EtOH and 40 ºC (1.47 g/ 100 g d.m.). Perhaps it is because the temperature and duration of the stirred tank extraction are not as high as during the Soxhlet extraction.

As the extraction temperature increases, the extraction yield increases with them, whether 96% ethanol or distilled water has been used as extraction solvents. The yields with distilled water were the lowest compared to the yields with ethanol, as these increased from 1.7 to 1.9 % (g/100 g dry matter) as the temperature increased. Comparing all the other results, there was a 1.4-fold increase as the extraction temperature increased from 40°C to 80°C. On the other hand, this did not occur with the 50% extraction where the extraction yield was the highest at 60⁰C temperature (4.04±0.77 %).

With respect to the progressive addition of water to the mixture, as it increases, the extraction yield increases until it reaches the maximum with 50% EtOH at 60⁰C (4.04± 0.77 %) thereafter the extraction yield decreases as the solvent acquires a greater proportion of water.

The lowest extraction yield obtained with 96% ethanol 1.1 % and it increased up to 4.04% obtained with 50% ethanol.

Three experiments were carried out at the middle point of extraction design in order to obtain the highest possible accuracy, obtaining an extraction yield of 4.04± 0.77 %.

The residue extracted from the first assay (ST1) with 96% EtOH at 40ºC was dried in the oven and re-extracted with 96% ethanol for another 3 hours at 40ºC. The residue from the first assay (ST1) with 96% EtOH at 40ºC was dried in the oven and re-extracted with 96% ethanol for another 3 hours at 40ºC. The extraction yield was 1.0% at this stage. The same steps were repeated again for 1 hour, achieving a yield of 0.7%. In total, the cumulative yield of the extraction was 3.14%, twice that obtained after the first phase.
As is well known, the lower the IC$_{50}$ factor the greater the antioxidant activity of the sample, in our case, the stronger antioxidant activity was measured in the presence of extract obtained with 50% EtOH at 80ºC with an IC$_{50}$ of 20.26 ±0.50 μg/ml. The extracts obtained with pure water showed the least antioxidant activities with the biggest standard deviations.

A comparison of the antioxidant activity of our sea buckthorn samples with synthetic antioxidants was made using the DPPH method, BHA showed the highest antioxidant activity (7.96 μg/ml), but the antioxidant activities of the sea buckthorn stalk extracts are also comparable with BHT antioxidant..

The induction time of the control sunflower seed oil was 2.5 ± 0.2 hours, and it increased when extract obtained with Soxhlet extraction using 96% ethanol added to the oil to 3.0± 0.1 h, which is an increase of 1.2-fold. Based on this, the sea buckthorn extracts obtained with 96% ethanol have a slight protective effect when mixed with vegetable oil at a concentration of 1%, resulting in an increase in stability of 1.2 times.

Based on these results, it can be concluded that value-added extract can be obtained from a waste, namely from sea buckthorn stalks using either 96% ethanol in Soxhlet extraction or 50% ethanol-water solution in the stirred tank apparatus. The extracts can be obtained at the highest yield (4.3%) in the stirred tank apparatus with strong antioxidant activity, which is comparable with that of synthetic antioxidant. While the extract obtained with 96% ethanol with Soxhlet extraction possessed a modest protection effect mixed it into sunflower seed oil in 1%. Further studies are required to map the individual compounds which might be responsible for these activities.
7. References

3. https://extramineral.lat/beneficios-de-espino-cerval-de-mar/
7. Gateway International Inc. Seabuckthorn and Health Summary 2009
11. Universidad de Granada. EXTRACCIÓN SÓLIDO-LÍQUIDO Y RECUPERACIÓN DEL DISOLVENTE: OBTENCIÓN DE ACEITE DE GIRASOL
20. Dr LM. Paucar Menacho. *Estabilidad a la oxidación de aceites y grasas por el método rancimat.*