KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

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Isolation of higher-added value components from bigroot geranium (*Geranium macrorrhizum*) and maral root (*Rhaponticum carthamoides*) by high-pressure extraction techniques

Master's Final Degree Project

Supervisor
dr. Michail Syrpas

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KAUNAS, 2018

KAUNO TECHNOLOGIJOS UNIVERSITETAS
CHEMINĖS TECHNOLOGIJOS FAKULTETAS

Aukštesnės pridėtinės vertės komponentų išskyrimas iš stambiašaknio snapučio (*Geranium macrorrhizum*) ir paprastojo rapontiko (*Rhaponticum carthamoides*) taikant aukšto slėgio ekstrakcijos metodus

Baigiamasis magistro projektas

Maisto mokslas ir sauga (kodas 621E40001)

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KAUNAS, 2018

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DECLARATION OF ACADEMIC INTEGRITY

4 June 2018
Kaunas

I confirm that the final project of mine, **Greta Grigoraviciute**, on the topic „**Isolation of higher-added value components from bigroot geranium (*Geranium macrorrhizum*) and maral root (*Rhaponticum carthamoides*) by high-pressure extraction techniques**“ is written completely by myself; all the provided data and research results are correct and have been obtained honestly. None of the parts of this thesis have been plagiarised from any printed, Internet-based or otherwise recorded sources. All direct and indirect quotations from external resources are indicated in the list of references. No monetary funds (unless required by law) have been paid to anyone for any contribution to this project.

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Grigoraviciute, Greta. Isolation of higher-added value components from bigroot geranium (*Geranium macrorrhizum*) and maral root (*Rhaponticum carthamoids*) by high-pressure extraction techniques. Master's Final Degree Project / supervisor dr. Michail Syrpas; Faculty of Chemical Technology, Kaunas University of Technology.

Study field and area (study field group): Technological Sciences, Food Technology.

Keywords: geranium, rhaponticum, SFE, PLE, antioxidants

Kaunas, 2018. 86 pages.

Summary

Geranium macrorrhizum is a perennial herbaceous plant of the *Geraniaceae* family, widely found in Central Europe. *Rhaponticum carthamoides* belongs to *Asteraceae* family, *Rhaponticum* Vaill. genus, it is widely consumed for centuries in Russia and Eastern Europe. Both plants are used in a folk medicine for its analgesic, antimicrobial, antioxidant, hypotensive, spasmolytic, astringent, cardiotonic, hepatoprotective properties and capillary sedative activities. This research was aimed to isolate and analyse bigroot geranium and maral root, grown in Lithuania, components by high-pressure extraction techniques.

In order to achieve this goal, two different high-pressure extraction techniques were carried out: supercritical carbon dioxide extraction and pressurized liquid extraction. Solid fat-free plant residues after supercritical carbon dioxide extraction were used for pressurized liquid extraction. SFE-CO_2 conditions were optimized using response surface methodology to maximize the extraction yield. PLE conditions were optimized using response surface methodology to maximize the extraction yield, total phenolic content and antioxidants activity.

SFE-CO2 geranium and maral root extract's total phenolic content was determined using Folin-Ciocalteu's technique. Antioxidant activity was measured by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, oxygen radical absorbance was measured by ORAC assay. Solid plants initial material and SFE-CO_2 residues were analyzed using same methods approaching QUENCHER procedure. PLE geranium extract's total phenolic content was determined using Folin-Ciocalteu's technique. Antioxidant activity was measured by $DPPH^{\bullet}$ (EC_{50}) and $ABTS^{\bullet+}$ assays.

Tentative chemical characterization of non-volatile compounds of geranium and maral root SFE-CO2 extracts were analyzed by UPLC/ESI-QTOF-MS, volatile compounds were analyzed using GCxGC/TOF MS. Characterization of phenolic profile of geranium PLE extract was performed using UPLC/ESI-QTOF-MS/MS.

Greta Grigoravičiūtė. Aukštesnės pridėtinės vertės komponentų išskyrimas iš stambiašaknio snapučio (*Geranium macrorrhizum*) ir paprastojo rapontiko (*Rhaponticum carthamoides*) taikant auškto slėgio ekstrakcijos metodus. Magistro baigiamasis projektas / vadovas dr. Michail Syrpas; Kauno technologijos universitetas, Cheminės technologijos fakultetas.
Studijų kryptis ir sritis (studijų krypčių grupė): Technologijų mokslai, Maisto technologijos.
Reikšminiai žodžiai: snaputis, rapontikas, ekstrakcija, SKE, PET, antioksidantai.
Kaunas, 2018. 86 p.

Santrauka

Geranium macrorrhizum – tai augalas, priklausantis *Geraniaceae* šeimai. Jis plačiai paplitęs Vidurio Europoje. *Rhaponticum carthamoides* priklauso *Asteraceae* šeimai. Jis nuo seno vartojamas Rusijoje ir Rytų Europoje. Abu augalai naudojami liaudies medicinoje dėl savo analgetinių, antimikrobinių, antioksidacinių, hipotenzinių, spazmus malšinančių, gerinančių širdies veiklą, sutraukiančių kraujagysles ir apsaugančių kepenis savybių.

Šio tyrimo tikslas - išskirti ir išanalizuoti *G. macrorrhizum* ir *R. carthamoides* aukštesnės pridėtinės vertės komponentus, taikant aukšto slėgio ekstrakcijos metodus. Buvo atlikti du skirtingi aukšto slėgio ekstrakcijos metodai: superkritinė anglies dioksido ekstrakcija (SKE-CO_2) ir pagreitinta ekstrakcija tirpikliais (PET). Kietoji frakcija po SKE-CO_2 ekstacijos buvo naudojama pagreitintai ekstrakcijai tirpikliais. SKE-CO_2 ekstakcijos sąlygos buvo optimizuotos siekiant maksimaliai padidinti ekstrakto išeigą. PET sąlygos buvo optimizuotos siekiant maksimaliai padidinti ekstrakto išeigą, bendrą fenolinių junginių kiekį ir antioksidacinį aktyvumą.

Stambiašaknio snapučio ir paprastojo rapontiko SKE-CO_2 ekstraktų bendras fenolinių junginių kiekis buvo nustatytas naudojant Folin-Ciocalteu metodiką. Antioksidacinis aktyvumas buvo matuojamas $DPPH^{\bullet}$ ir $ABTS^{\bullet+}$ tyrimais, deguonies radikalų absorbcija buvo matuojama ORAC tyrimu. Kietoji augalų pradinė medžiaga ir liekana po SKE-CO_2 ekstakcijos buvo analizuojami naudojant tuos pačius metodus, taikant QUENCHER procedūrą. *G. macrorrhizum* pagreitintos ekstakcijos ekstrakto bendras fenolinių junginių kiekis nustatytas naudojant Folin-Ciocalteu metodiką. Antioksidacinis aktyvumas buvo matuojamas $DPPH^{\bullet}$ (EK_{50}) ir $ABTS^{\bullet+}$ tyrimais.

Stambiašaknio snapučio ir paprastojo rapontiko SKE-CO_2 ekstraktų preliminari cheminė analizė atlikta naudojant UPLC/ESI-QTOF-MS metodiką. Preliminari lakiųjų junginių analizė atlikta naudojant GCxGC/TOF MS metodiką. *G. macrorrhizum* PET ekstraktai preliminariai išanalizuota naudojant UPLC/ESI-QTOF-MS/MS metodika.

LIST OF ABBREVIATIONS

ABTS●+	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation
ANOVA	Analysis of variance
AS	Analytical scale
ASE	Accelerated solvent extraction
AUC	Area under curve
CCD	Central composite experimental design
CIAL	Instituto de Investigación en Ciencias de la Alimentación
CSIC	Consejo Superior de Investigaciones Científicas
DAD	Diode-array detector
DPPH●	2,2-diphenyl-1-picrylhydrazyl radical
EO	Essential oil
ESI-QTOF	Electron Spray Ionization - Quadrupole Time of Flight
FID	Flame ionisation detector
GAE	Gallic acid equivalents
GC	Gas chromatography
GRAS	Generally Recognized as Safe
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
LC	Liquid chromatography
MCF-7	Breast cancer cell line
MDR	Multidrug resistence
MeSH	Medical Subject Headings
MS	Mass spectrometry
ORAC	Oxygen radical absorbtion capacity
PBS	Phosphate buffered saline
PDA	Photodiode Array
PHWE	Pressurized Hot Water Extraction
PLE	Pressurised liquid extraction
PS	Pilot scale
R^2	Determination coefficient
RSC	Radical scavenging capacity
RSD	Residual standard deviation
RSM	Response surface methodology
SC-CO2	Supercritical carbon dioxide
SFE	Supercritical fluid extraction
SFE-CO2	Supercritical carbon dioxide extraction
SLE	Solid liquid extraction
SLE-ACN	Solid liquid extraction using acetone
SLE-Et	Solid liquid extraction using ethanol
SLE-He	Solid liquid extraction using hexane
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UPLC	Ultra-high performance liquid chromatography

VOCs	Volatile Organic Compounds
VS	Versus

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INTRODUCTION

In the past decade, the study of nutritional and biological activities of food related plants has increased. Moreover, active constituents such as phenolic, heterocyclic compounds or terpenoids that can be derived from plant material have gained considerable importance because of its potential benefits for health. Studies have shown that these compounds can act as antioxidants [1], [2], [3]. By scavenging free radicals, they can significant prevent oxidative stress of substrate, causing the damage of DNA. Thus, antioxidants can lower incidence of cancer, inflammation, rheumatoid arthritis, atherosclerosis, etc [4], [5]. Moreover, antioxidants can be added to food to lower oxidation of lipids, preserve quality, colour, flavour and safety [6].

Geranium macrorrhizum is a perennial herbaceous plant of the *Geraniaceae* family, widely found in Central Europe. It is commonly known as 'big root geranium' and 'rock crane's-bill'´ [7]. *Rhaponticum carthamoides* which belongs to *Asteraceae* family, *Rhaponticum* Vaill. genus, it is widely consumed for centuries in Russia and Eastern Europe. R. carthamoides is also known as Maral root, *Rhaponticum* or lauzea [8].

This research was aimed to isolate and analyse bigroot geranium and maral root, grown in Lithuania, components by high-pressure extraction techniques. In order to achieve this goal, the following objectives were set:

1. To determined chemical profile of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots (nitrogen content, oil content, water content, ash content);

2. To optimize supercritical carbon dioxide extraction (SFE-CO_2) conditions in order to obtain the highest yield of *G. macrorrhizum* leaves lipophilic fraction, evaluating the effect of three selected factors by response surface methodology (RSM). Moreover, to obtain SFE-CO_2 extracts of *G. macrorrhizum* roots and *R. carthamoides* roots.

3. To measure total phenolic content and *in vitro* radical scavenging capacity of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts, starting plant materials and solid residues after SFE-CO_2 extraction.

4. To characterize volatile profile of *G. macrorrhizum* leaves SFE-CO_2 extract under optimized conditions by GCxGC/ TOF MS and to determine the changes in germacrone content under various extraction conditions;

5. To characterize non-volatile compounds of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts using UPLC/ESI-QTOF-MS;

6. To apply consecutive solid-liquid extraction (SLE) method with different polarity solvents for non-polar and polar fraction isolation for *G. macrorrhizum* leaves, roots and *R.*

carthamoides roots and to compare efficiencies of conventional and high-pressure extraction techniques.

7. To optimize pressurized liquid extraction (PLE) in order to obtain the highest yield, total phenolic content and *in vitro* antioxidant activity of polar fraction from *G. macrorrhizum* leaves residues after SFE-CO_2, evaluating the effect of two selected factors by response surface methodology (RSM).

8. To characterize non-volatile profile of *G. macrorrhizum* PLE extract under optimal conditions by UPLC/ESI-QTOF-MS/MS.

1. LITERATURE REVIEW

1.1. *Geranium macrorrhizum*

1.1.1. General characteristics

Geraniaceae family comprises 5 to 7 genus and around 830 species. The most important genus is *Geranium*. Due to moist and shaded environments, this genus mainly occurs through the northern hemisphere, in temperate and mountainous regions [9].

G. macrorrhizum is a perennial herbaceous plant of the *Geraniaceae* family. *G. macrorrhizum* can widely be found in Central Europe. Unfortunately, it doesn´t grow in Lithuania, except botanical gardens, flower beds and rockeries. *G. macrorrhizum* is commonly known as 'big root geranium', 'Bulgarian geranium' and 'rock crane's-bill'. This plant has a big rhizome, five-lobed aromatic leaves and pale pink flowers. *G. macrorrhizum* is illustrated in ***Figure 1.1.***

G. macrorrhizum is highly valued in folk medicine for the treatment of stomach disorders in form of infusion as well as an aphrodisiac. Historically it has been used in the treatment of dysentery, heavy menstrual flows, hemorrhoids. Also, extracts of the plant have analgesic, antimicrobial, antioxidant, hypotensive, spasmolytic, astringent, cardiotonic, hepatoprotective properties and capillary sedative activities. Currently, French traditional medicinal community treats diarrhoea, gasctric ulcers, diabetes, gallbladder problems, liver problems, urinary stones, jaundice and sterility with *Geranium* oil. Furthermore, in Chinese traditional medicine it is known as a toxins reliever. [10] *Geranium* oil can reduce post-herpetic neuralgia pain of shingles. [11] Because of *G. macrorrhizum* beautiful pink flowers, it is also known for ornamental purpose. [12] Ornamental varieties have been developed to tolerate low water and light, also stand bitter cold weather. *G. macrorrhizum* has received much attention in the last years due to its phytochemical properties. [13] *G. macrorrhizum* is a natural source of antioxidant compounds such as gallic acid, ferulic acid, chlorogenic acid, etc. [14] Essential oil (EO) from the *G. macrorrhizum* inhibits growth of *Bacillus subtilis* [15]. The major compound of *G. macrorrhizum* EO, sesquiterpene germacrone, has dose-dependently antiviral effect against several influenza viruses. [16]

However, due to the limited availability of this plant from wild-growing populations and no references to cultivation, there are difficulties in producing essential oil of *G. macrorrhizum.* [17] This species are found in very small amounts, thus oil from wild-growing plants is expensive.

Figure 1.1. Illustration of *G. macrorrhizum*. Adapted from *Curtis's Botanical Magazine* **[18]**.

1.1.2. Chemical profile and bioactivity

G. macrorrhizum is highly valued in folk medicine for the treatment of stomach disorders in form of infusion as well as an aphrodisiac. Extracts of the plant have analgesic, antimicrobial, antioxidant, hypotensive, spasmolytic, astringent, cardiotonic, capillary sedative activities and hepatoprotective properties [19].

According to Ognyanov *et al*. the EO of aerial parts of *G. macrorrhizum* consinsts of approximately 50% Germacrone ***(Figure 1.2-3)*** [20]. Moreover, it contains two well-known monoterpenoids – geraniol (***Figure 1.2-1***) and β-citronellol (***Figure 1.2-2***), along with some other sesquiterpenes such as α-elemene (4), α-curcumene (5) (***Figure 1.2-4, 2-5,*** respectively). Mihailov *et al*. reported the presence of tannins, flavonoids, phenolic acids and waxes. [21]

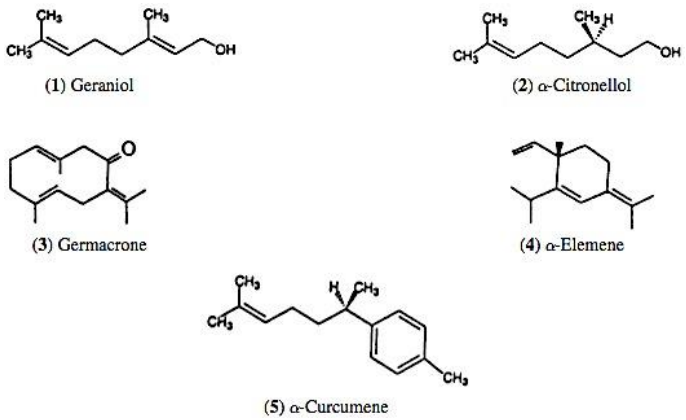

Figure 1.2. Essential oil components of aerial parts of *Geranium*. Adapted from *Structure of germacrone.* by Ognyanov *et al*. **[20]**

According to Radulović *et al*. [15] the main sesquiterpene in EO of *G. macrorrhizum*'s rhizomes is δ-guaiene.

Sesquiterpenes of *Geranium machrorrhizum*. Germacrone, one of the main sesquiterpenes in essential oil of aerial parts of *Geranium machrorrhizum*, in a dose-dependent manner showed antiviral activity against the H1N1 and H3N2 influenza viruses and the influenza B virus. Studies showed that MDCK and A549 cells treated with this sesquiterpene decreased RNA synthesis and the production of infectious progeny viruses in the viral protein expression. Furthermore, in the attachment step and the early stages of the viral replication cycle germacrone exhibited an inhibitory effect. Studies showed, that this compound can reduce the viral titre in the mices lungs and protect it from lethal infection [16]. Recently it was reported that Germacrone can arrest cell cycle, thus it might be a treatment of human hepatoma cell lines HepG2 and Bel7402. Germacrone can regulate the expression of protein related G2/M cell cycle and apoptosis. Thus, this chemical compound might be a new liver cancer potent chemo preventive drug candidate [22]. At present, resistence in chemoterapeutics is the major reason of chemoteraphy failure. Recent studies have been shown that germacrone can be a novel multidrug resistence (MDR) agent in chemotherapy of breast cancer [23]. α-elemene, found as another component in the essential oils of *G. macrorrhizum* [20], can be a natural drug again cancer. Zhu *et al*. reported that this compound can inhibit glioblastoma growth [24]. Moreover, another sesquiterpene of *G. macrorrhizum*, α-curcumene, has anti-inflammatory activity that was reported by Lenfeld *et al*. Their studies were done on rats with a carrageenan and formalin oedema [25]. Scwob *et al*. screened the antimicrobial activity of α-curcumene. Results showed that this sesquiterpene has a high activity against *Saccharomyces cerevisiae* [26].

Monoterpenoids of *Geranium machrorrhizum*. Monoterpenoid geraniol (GE), that occurs in Geraniceae plants [27], can be used as a treatment against diabetes. Studies showed that key enzymes of glucose metabolism can be ameliorated by geraniol [28]. GE can inhibit biofilm formation in *Staphylococcus epidermidis*. Therefore, it is a potent candidate to control biofilm-mediated infection of this microorganism [29]. Another monoterpenoid, namely β-citronellol, has repellent properties against the ticks *Rhipicepjalus sanguineus sensu lato* and *Amblylomma scultptum* and can protect humans against diseases transmitted by these two species [30]. Furthermore, recent studies showed that this chemical compound can have an inhibitory effect on cytokine production and degranulation by mast cells. It leads that this monoterpenoid can be a treatment for allergic diseases [31].

1.2. *Rhaponticum carthamoides*

1.2.1. General characteristics

Rhaponticum carthamoides belongs to *Asteraceae* family, *Rhaponticum* Vaill. genus. *R. carthamoides* is also known as Maral root, *Rhaponticum* or lauzea. This plant traditional name (Maral root) came from the maral deer who fed on it. It is worth mentioning that it has been researched by scientific orgnizations since the year 1929.

R. carthamoides is a perennial herceous plant with rarely branched stems. *Rhaponticum* plant leaves can be up to 60 cm long and are deeply incised with pointed edges, they similar to leaves of pinnatifid. In the apex of the stem is the inflorescence. Anchene, the fruit of *R. carthamoides*, has hairy pappus. *R. carthamoides* is illustrated in ***Figure 1.3.*** *R. carthamoides* naturally grows in the mountains of South Siberia. Maral root is widely consumed for centuries in Russia and Eastern Europe. *Rhaponticum* is also distributed in Xinjiang, China and is used in Chinese folk medicine for treatment of anepithymia, prostration and hypertension. In Russian and Siberian medicine *R. carthamoides* is widely used against overstrain and weakness, as well as support physical performance and to promote normal energy levels, especially after illness [8]. In addition, it is known as a supporter for stamina and healthy libido for men and women. Studies have shown that cultivated forms of *Rhaponticum* lacks important active substances (sesquiterpene lactones, flavonoids, polyamines) and therefore are less effective than wild form [32].

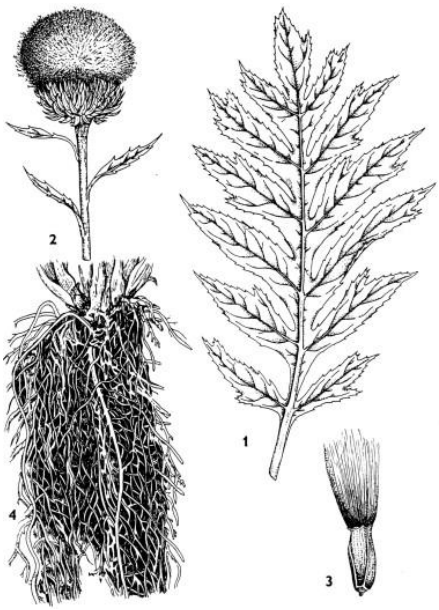

Figure 1.3. Line drawings of *R. carthamoides*: 1. Leaf. 2. Inflorescence. 3. Fruit 4. Roots **[33]**

1.2.2 Chemical profile and bioactivity

Previously a lot of different compounds were isolated from different anatomical parts of *R. carthamoides*. The main bioactive compounds of the *R. carthamoides* plant are polyphenols (flavonoids and phenolic acids) and steroids, particulary ecdysteroids [34]. The plant is also rich in polyacetylenes, sesquiterpenes lactones, triterpenoids glycosides, polyamines, lignans (trachelogenin and carthamogenin) and terpenes [8]. The roots of *R. carthamoides* are great source of polyphenolic compounds, mainly caffeoylquinic acids, their derivatives and flavonoids [8], [35].

Ecdysterones, one of main compounds in *R. carthamoides*, are polyhydroxylated sterols that control cell proliferation and growth. In plant, these compounds act as natural insecticides [32]. Ecdysterone content in *R. carthamoides* is around 0.7% in the roots and 2% in the seeds. 20-hydroxyecdysone is one of the major ecdysterones in *R. carthamoides* plant. In addition, Skiba and Weglarz reported phenolic acids of *R. carthamoides*. They detected benzoic acid, m-Hydroxybenzoic acid, p- Hydroxybenzoic acid, Salicylic acid, Gentisic acid, Gallic acid, Syringic acid, o-Coumaric acid, sinapic acid, o-Hydroxyphenylacetatic acid and p-Hydroxyphenylacetatic acid [36]. Studies report that *R. carthamoides* possess haemorheological and central nervous system stimulating activity [37]. Ecdysterones have neurotrophic activity. Its mechanisms are partially unknown, but this compound is associated with Akt signaling and inhibition of the pro-apoptotic enzyme caspase-3 [38], [39].

R. carthamoides and its extracts have high antioxidant and antimicrobial activity. These properties can be attributed to a wide range of polyphenols such as flavonols and flavonoids (quercetin, rutin, epicatechin etc.) in the plant [40], [8]. Polyphenolic compounds can arrest cancer cell cycle, enhance the expression of apoptotic genes and promote apoptosis of cancer cells, in addition to inhibit angiogenesis and metastasis [41], [42], [43]. Scala *et al* reported that 80 % methanol extracts of Maral roots demonstrated anticancer activity. It can induce apoptosis in grade IV human glioma cells through the loss of mitochondrial membrane potential and regulate of apoptosis-related protein expression. [44] In addition, leaf extracts have cytotoxic effect on MCF-7 human breast cancer cell line [45]. Skala *et al*. also reported caffeoylquinic acids and their derivatives in *R. carthamoides*[46]. These compounds are known to have chemopreventive and chemotherapeutic effects [47].

1.3. High pressure and sustainable extraction methods

1.3.1. Sustainable extraction techniques

In recent years, the control of harmful substances has been increased and protection of environment and consumers are the main challenges for all producers. Industries must be more ecologic, modern, economic and innovative if they still want to be competitive in the market. Nowadays, in the multidisciplinary area of applied biology, chemistry and technology the design of green and sustainable extraction methods of natural products is a very important research topic [48]. Currently, most organic solvents which are used in product extractions can have a negative impact on people, environmental pollution, contribute to climate change, greenhouse effect, moreover they are flammable and volatile. Environmental, safety and economical aspects are pushing production industry to turn towards greener alternatives in their processes. New regulations for petrochemical solvents and Volatile Organic Compounds (VOCs) have a progressive impact. All the producers that are using organic solvent must show the lack of risk in their production processes and demonstrate the safety of ingredients for traces of the organic solvents [49]. Modern and conventional extraction techniques help to maximize the quality of results and minimize the costs in a shorter time. Also, by using "greener" extraction processes obtained extracts are less harmful and have lower impact on the enviroment and people.

1.3.2. Supercritical fluid extraction (SFE)

Supercritical fluid extraction is an extraction method that can selectively remove an analyte from a solid, semi-solid matrix or a liquid by using supercritical fluids. This technique has been developed to make sample preparation faster and less solvent intensive. SFE-CO_2 is focused on designing "greener" processes which do not have a harmful effect for environment and people. [49] SFE-CO_2 is mainly used for bioactive compounds extraction from natural sources. This extraction technique finds applications in food, pharmaceuticals and cosmetics industries.

Apparatus: Supercritical fluid extractor system consists of a solvent pump, modifier pump (if necessary), an extraction cell, one or more fractionation cells and valve. (***Figure 1.4***). Basically, solvent pump delivers the fluid through the system. Often extraction cell and fractionation cells are equipped with pressure and temperature controls for stepwise depressurization/fractionation. Also, in a supercritical fluid extractor system recycling equipment can be installed. The cost of

gaseous CO_2 to supercritical or liquid state is high so it´s important to recycle fluid which was used as a solvent [50] [51].

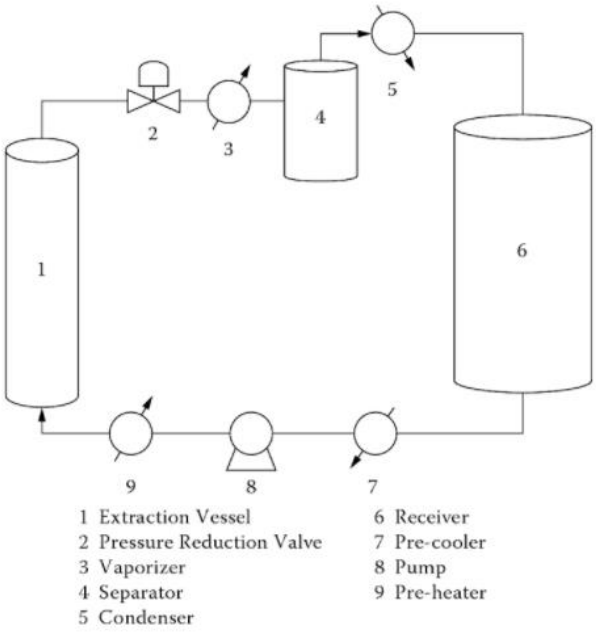

Figure 1.4. Schematic diagram of supercritical CO_2 extraction system. Adapted from Martinez **[52]**.

Operation: A fluid becomes supercritical when it is forced to a temperature and pressure above its critical point (***Figure 1.5***). At the critical point, the viscosity of super critical fluid is similar to gas and density is similar to a liquid, but its diffusivity acts as intermediate between fluid and liquid (***Table 1.1***). Thus, supercritical fluid has similar solvating power and density as a liquid but can be compressed as a gas.

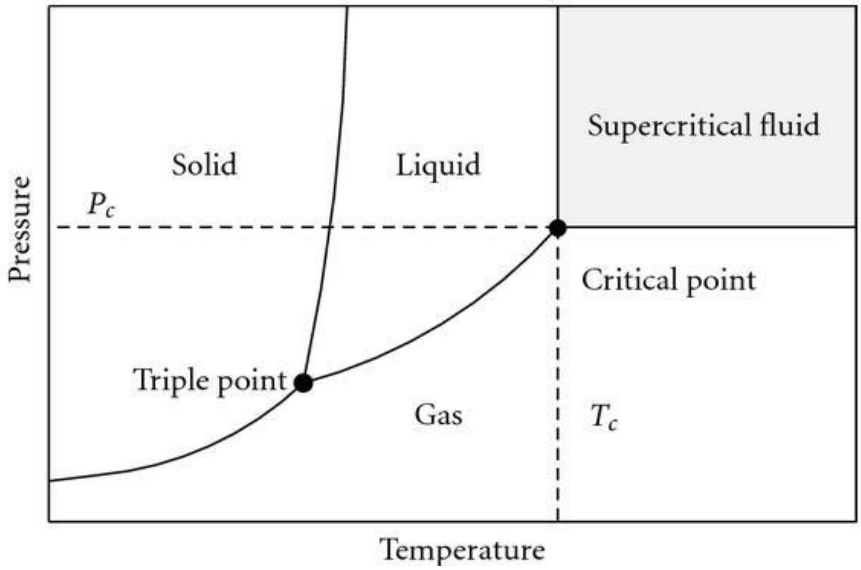

Figure 1.5. Pressure-temperature phase diagram of supercritical fluid. Adapted from Liao *et al* **[53]**.

At room temperature, carbon dioxide is a gas, thus, the extract is solvent-free. In the SFE design with recycling, CO_2 is cooled, recompressed and return to the storage.

Table 1.1. Physicochemical properties of gases, liquids and supercritical fluids

Fluid state	Viscosity (μ, g s/cm)	Density (ρ, g/cm3)	Diffusivity (DAB, cm2/s)
Gas T = 21 °C, p = 1 atm;	10^{-4}	10^{-3}	10^{-1}
Liquid T = 15–30 °C, p = 1 atm;	10^{-2}	1	$<10^{-5}$
Supercritical $T = T$c, $p = p$c;	10^{-4}–10^{-3}	0.3–0.8	10^{-3}–10^{-4}

Solvents: Several solvents are proposed for SFE such as butane, propane, dimethyl ether. Even though these solvents are known as toxic, the required amount for SFE is much smaller than the amount needed at low pressure extraction techniques [2]. However, none of these solvents fulfil the principles of "green" technique as carbon dioxide does. CO_2 is non-flammable and non-toxic, its critical conditions are easily achievable. Moreover, carbon dioxide for the use in food industry is Generally Recognized as Safe (GRAS).

Modifiers: As CO_2 has a low polarity, polar modifiers can be introduced into the system at low amounts (1-10 %) to change the solvating properties according to interested analytes such as polar bioactive compounds (phenolic acids, flavones, anthocyanins, etc) [54]. The most common modifier in super critical carbon dioxide is ethanol. Furthermore, acetone, methanol and even small amounts of water can also be applied in this extraction technique. Commonly, modifiers are added to the extraction cell directly, also they can be added to CO_2 flow using another pump [55].

Temperature and pressure: Temperature and pressure have a strong influence on solvent properties such as viscosity, density, diffusivity. It leads that temperature and pressure is related to solubility of analytes in the sample. Normally, for lipids isolation from plants, microalgae, algae, oils or dairy products the range of the temperature is from 40 to 50 ºC and pressure in the range from 10 to 30 MPa is used [56].

Efficiency: In order to achieve better efficiency, the side of the sample particle sizes and crushing degree can be modified. Particle sizes are very significant factor for mass transfer and extraction yield. Also, to increase extraction rate and avoid clogging, dispersing agents can be used. Extraction efficiency can also be enhanced using modifiers added to the primary fluid [57].

Develpoment: SFE-CO_2 is commonly applied weakly polar or non-polar compounds such as carotenoids, fatty acids, triglycerides, etc. extraction [58] [59] [60]. One of applications of SFE-CO_2 in food industry is fat removal from food [61]. According to Rozzi and Singh [62] as

well as Curent contents database [63], the main applications of supercritical fluid extraction are food and agriculture (32 %) and fuel industry (24 %). According to Food Science and Technology Abstracts (FSTA) database [64] and the number of papers which was published in the Food Science and Technology field, the main SFE application are flavor and natural compounds (37 %) , oils and essential oils (EOs) (26 %). In the food sector, essential oils extracted using SFE-CO_2 most often used as food flavourings. [48]

Advantages: The main advantages of supercritical carbon dioxide extraction among other extraction techniques is higher extraction efficiency (shorter time and higher yields), wide selectivity, absence of toxic solvents, CO_2 generally recognized as safe (GRAS). Moreover, several studies have compared antioxidant activity between extracts obtained by SFE and other extraction techniques. Fadel *et al*. reported that SFE extracts has higher antioxidant activity than extracts obtained by hydrodistillation (HD) [3]. Stashenko, Jaramillo & Martinez isolated more bioactive compounds of *Lippia alba* by SFE than using hydro distillation and micro-wave hydro distillation extracts. Antioxidant activity of SFE extracts was also greater than in extracts of hydro distillation [65].

Disadvantages: Although the cost of SFE equipment has decreased in the recent years [66] still the main drawback of supercritical CO_2 extraction is high initial investments [67] and high-operation costs (high energy consumption) due to application of the high pressure. Furthermore, since SFE systems are using high pressures, a lot of attention should be given to safety parameters. The explosion of pressure vessel could cause harm on people and damage buildings [68]. Also, an important disadvantage is less effective solute solubility in supercritical solvents in comparison to organic solvents. Likewise, in principal CO_2 is non-polar solvent, it cannot dissolve polar molecules. Here the solubility of polar compounds can be increased with addition of modifiers, but it increases capital costs and complicates system thermodynamics. [56] Moreover, the addition of modifiers causes poor selectivity, for example waxy material can be extracted with wanted compounds. [69]

1.3.3. Pressurized liquid extraction (PLE)

Pressurized liquid extraction is an environmentally friendly solid-liquid extraction technique that is based on using organic solvent at high pressure and temperature (always below solvents crucial points). Richter *et al.* first described PLE in 1996 [70]. Pressurized liquid extraction is suitable to extract a wide range of solutes, from polar to non-polar. It has been used as an efficient method to obtain phenolic compounds from natural samples. In comparison with other extraction techniques which are carried out at near room temperature and atmospheric pressure, using

elevated temperature and pressure enhance the extraction performance [34]. This technique is also known as accelerated solvent extraction (By Dionex Corporation), enhanced solvent extraction and pressurized solvent extraction. When water is an extractant solvent, this method refers to Pressurized Hot Water Extraction (PHWE), superheated water extraction and sub-critical water extraction.

Apparatus. A schematic diagram of a pressurized liquid extractor is shown in ***Figure 1.5***. Normally, it consists of solvent pump, extraction cell, heated oven, collection vial, nitrogen tank and valves. Most of the extractions cells are made of 316L stainless steel.

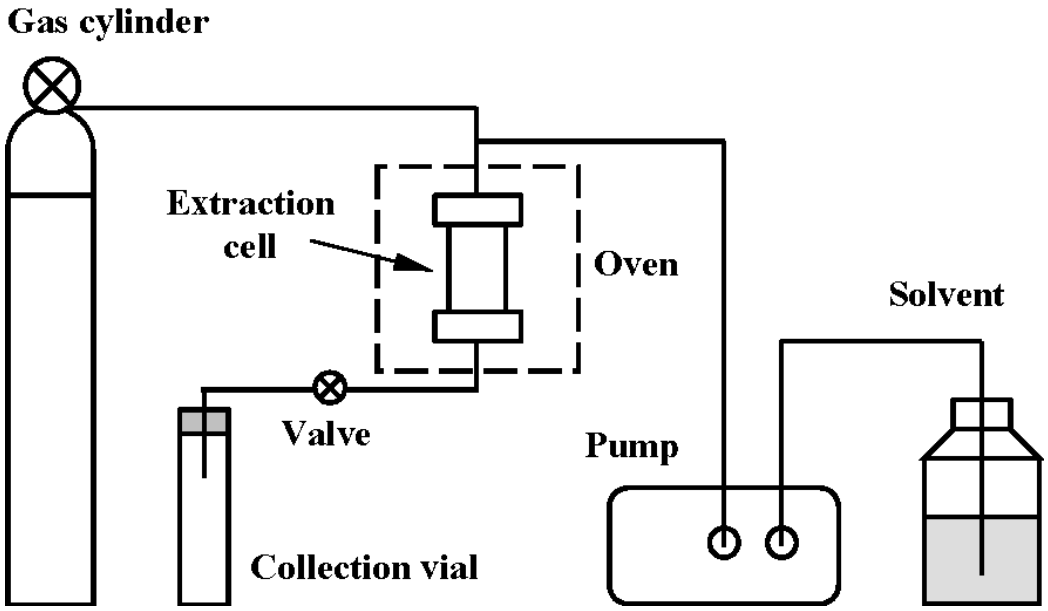

Figure 1.5. Schematic diagram of pressurized liquid extraction system. Adapted from Lundstedt & Avhandling. **[71]**

Operation: Firstly, the selected solvent (water, ethanol, etc) is pumped to fill the cell that contains sample. Afterwards, the cell is pressurized and heated for predetermined extraction time, which is typically 10-15 min. Next a few millilitres of fresh solvent are dynamically pumped through the extraction chamber and connective tubes. At the end of the last extraction cycle, to guarantee the complete wash off the solvent from the cell and the tubing into the collection vial, an inert gas (nitrogen) is purged for 1-2 minutes.

Dynamic and static modes: PLE can be carried out by dynamic (flow), static and in the combination by both modes. In the dynamic mode, the extraction solvent is continuously pumped through the sample vessel. In static set-up, the whole process consists of one or more extraction cycles with the replacement of the between cycles. To use the combination of static and dynamic in the same run is the most widely used mode in pressurized liquid extraction.

Temperature and pressure: As elevated temperature and pressure are always below solvents crucial points, normally the range of the temperature is between 50-200 ºC and pressure

is between 35-200 bars, respectively. [72] Solvents above their atmospheric boiling point have lower surface tension and viscosity that enhances mass transfer and solubility of analytes. Hawthorne and Miller reported that temperature is more important to extraction efficiency than pressure [73]. Elevated temperature enhances sample wetting by the solvent.

Solvents: Main aspects in the choice of the solvent is desired analyte solubility. Polar analytes dissolves in polar solvent and likewise non-polar analytes dissolves in non-polar analytes [74]. The analyte of interest should have a very high solubility in the chosen solvent, while other compounds ideally should have no or have as low as possible. Also, sustainability, economy and safety aspects should be considered prior to the choice of solvent. The most preferable solvents are non-harmful and less toxic.

Commonly, in PLE water or ethanol are used as solvents for the extraction of bioactive compounds from foods and herbs. Although, methanol, n-hexane, dichloromethane, propane, ethyl acetate, acetone, surfactants and ionic liquids can also be applied [75] [76] [77].

Hawthorne and Miller were first who introduced water in pressurized liquid extraction system. Commonly, it is called Pressurized Hot Water Extraction (PHWE) [73]. The water at the temperature between 100 ºC and 374 ºC is subcritical water [78]. However, by increasing temperature, pressure should be increased to keep the liquid phase of water. I.e. for the 200 ºC temperature 15 bar of pressure are needed, for 300 ºC 85 bar. Otherwise, superheated steam will be formed if the pressure will be lower. [79] Nevertheless Petersson *et al*. studied anthocyanins from red onion and reported that there is a risk of antioxidant degradation [80] which was also shown by Co *et al*. on birch bark [81]. Despite the fact that water is mainly used in natural water-soluble compounds extraction such as acids, sugars, proteins, [48] Kim *et al* analysed and reported that increasing temperature, leads to a substantial decrease in polarity, viscosity and surface tension of water [82] Thus, by increasing temperature, water could be suitable to extract polar, moderately polar and non-polar compounds. Although ethanol is flammable and potentially explosive, it is one of the most common bio-solvents. This solvent is used because it is easily available in high purity and has complete biodegradation.

Extraction enhancers: To facilitate the efficiency of the extraction filter papers, dispersing and drying agents can be used depending on the sample matrix. The aggregation of sample can affect extraction efficiency, in this case inert materials such as diatomaceous earth or sand can be used. During the extraction, diatomaceous earth can be used for dispersing and dehydrating agents [83]. Dispersing samples is recommended when samples contain very small particles, it tend to compact the extraction cell outlet. Dispersing agents are also used to fill up the vessel and reduce the solvent consumption by reducing vessel volume.

Efficiency: To achieve optimal efficiency, temperature, pressure, solvents, mode of extraction (dynamic or static), extraction time, samples particles size and extraction enhancers can be changed. Reducing samples particles size and increasing temperature can prevent extraordinary long extraction times. Moreover, solubility of analyte in the extraction solvent increases efficiency of extraction [84]. Using solvent mixtures can enhance extraction yield by increasing interaction with analyte. [85] The extraction process will ultimately enhance by using diatomaceous earth to absorb water from the sample matrix, since the presence of moisture can negatively affect the efficiency of extraction.

Advantages: The main advantages of pressurized liquid extraction are shorter extraction time and reduction in the amount of solvent used. [86] Also, in contrast to traditional solvent extractions, process is automated, sample is kept in light and oxygen-free environment.

Disadvantages: Normally, pressurized liquid extraction operates at high temperatures, such as 110-150 ºC. At these temperatures thermally labile analytes, for example bioactive compounds (such as anthocyanins), can degrade [87] Thus, it can lead to lower extraction yield and lower bioactivity. Since PLE system is working under high pressures, a lot of attention should be given to the importance of safety. As it was mentioned in SFE section, explosions of pressure vessels could cause harm. Moreover, as high pressures and temperatures are required, PLE setup is relatively more expensive than traditional extraction methods [88].

1.3.4. Processing and technology of *G. macrorrhizum*

In folk medicine, *Geranium* is mostly used as an essential oil. It is one of the most expensive EO in the flavouring industry [89]. The most common process to get an oil of *Geranium* is steam distillation. [10] This extraction method uses high temperature (over 100 °C), therefore thermal degradation can occur. Because of degradation of high-value substances, the final product is not fully presenting true essence of *Geranium*. As reported in the literature oil recovery from *Geranium* using steam distillation is 0.15 %. [10]. Thermal degradation of compounds can explain their low extraction yield as well as low bioactivity. Farukh *et al.* found that its essential oil of *Geraniceae* family plant is rich in citronellol, linalool, menthone, geraniol and caryophyllene oxide [90].

Some studies were done on supercritical carbon dioxide extraction (SFE-CO_2) of *Geranium* [91]. Peterson *et al*. measured the changes of oil yield by changing three factors – temperature, pressure and flow rate of CO_2. Results showed that high pressure (300 bar), low temperature (40 ºC) and low flow rate of CO_2 (2.0 mL min^{-1}) is the optimum conditions for extraction SFE-CO_2. Even though under these condition the yield was not the highest, but wax

content was lower than using low pressure (100 bar), high temperature (70 ºC), and high flow rate of CO_2 (4 mL min^{-1}). The main constituents of *Geraniceae* family plant SFE-CO_2 extract have been reported to be citronellol, citronellyl formate and geraniol [91].

Currently, there is a lack of data in the literature of pressurized liquid extraction (PLE) on *Geranium* plant. PLE may be directed to compounds that have high and medium polarity extraction, depending on the solvent used in the process. Moreover, there are no reports on the bio-refinery study of *G. macrorrhizum*.

1.3.5. Processing and technology of *R. carthamoides*

In traditional medicine *R. carthamoides* can be used as a pure sample or extract. In a pure sample form, they can be used both fresh and dried. Different procedures can be applied for extraction of *R. carthamoides*. The most common techniques are solid-liquid extraction (SLE), Sohxlet extraction, supercritical fluid extraction (SFE), ethanol-modified SFE, pressurized liquid extraction (PLE), etc. [92]. By comparing different extraction techniques and obtained extracts, previous reports showed that supercritical fluid extracts (SFE) have the highest antioxidant activity [92], [93].

1.3.6. Bio-refinery concept

Nowadays, the demand of natural products extracts is increasing. This situation is leading to the overuse of natural plant resources, some species started to be very rare or even extinct. [49]. By shifting the focus from getting single product, bio-refinery concept can by applied [94], [48]. Currently, uncontrolled harvesting in a large-scale of natural plant resources carries the risk of overuse. Thus, "Bio-refinery" concept is becoming widely accepted. [48] Bio-refinery can be considered as a facility that makes the conversion of biomass into chemicals, biomaterials and energy with the aim to minimize the waste and maximize the value of the raw material by making the process economically feasible [95]. In a bio-refinery process high value, but low volume products such as pharmaceuticals, nutraceuticals, cosmetics or low value but high-volume products such as biodiesel, bioethanol are produced from biomass [49]. Pressurized fluids have been used for different plant by-products for phytochemical extractions. Even so, it's largely unexplored field and it gives many research opportunities to produce several products from agriculture and forest residual material. [48] Now product development is focused on designing effective green bio-refinery processes which do not have a harm effect on people, environment and it will promote recycling reuse of residues of food related products.

Sources states that around 500 million of *Geranium* plants are harvested in 9980 ha every year for oil production [96]. For example, in the case of steam distillation use to produce EO, the recovered amount of oil is 0.15% [97]. It leads that every year around 499 million of geranium plants are wasted along with the used land for harvesting. Peterson *et al*. reported that by using SFE-CO2 for geranium oil extraction the yield is much higher (2.53%) than the yield obtained by steam distillation.

Up to now, there are no studies focusing on *Geranium* bio-refining. However, previous studies of other food-related waste material [98] confirm bioactivity of SFE-CO_2 by-products. Considering possible bioactivity of defatted geranium material, bio-refinery concept can be applied here by the application of pressurized liquid extraction to the residues obtained by super critical carbon dioxide extraction of geranium.

2. MATERIALS AND METHODS

2.1. Plant material

G. macrorrhizum leaves, roots and *R. carthamoides* roots in 2016 summer were collected from Kaunas Botanical Garden of Vytautas Magnus University in Lithuania and dried. Dried plant material was kept in in the dark, well-ventilated storage place at ambient temperature (~20 °C). For further experiments, dried plants were grinded in a centrifugal high-speed roto mill at 8000 rpm (Restch ZM 200, Resch GmbH, Haan, Germany) into the fraction of 0.2 mm. Grinded material was stored in well closed dried glass jars, in a dark well-ventilated storage place at ambient temperature (~20 °C) until future procedures and experiments.

2.2. Chemicals and reagents

Analytical/HPLC grade hexane, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$, 99 %), catalytic tablet (K2SO4, CuSO4), conc. H_2SO_4, NaOH, H_3BO_4, Na_2CO_3, 2,2-Diphenil-1-picrylhydrazyl hydrate ($DPPH^{\bullet}$, 99%), 3,4,5-trihydroxybenzoic acid (gallic acid, 99 %), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97 %), germacrone standard (≥99.0%) (*Sigma-Aldrich Chemie, Steinhein, Germany*), analytical/HPLC grade methanol (*Sigma-Aldrich, Poole, UK*), hexane (*PENTA Chemikalien, Mainaschaff, Germany*), Folin-Ciocalteu's phenol reagent 2M, Fluorescein (FL) (*Fluka Analytical, Bornem, Belgium*), Toshiro indicator, HCl (33-38 %, *Chempur, Piekary Slaskie, Poland*), nitrogen liquid (*AGA SIA, Riga, Latvia*), carbon dioxide, nitrogen gases (99.9%, *Gaschema, Jonava region,*

Lithuania), cotton-wool (*Bella-cotton, Poland*), microcrystalline cellulose 20 μm (*Sigma-Aldrich, St. Louis, MO, USA*), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (*Lach-Ner, Brno, Czech Republic*), Na_2HPO_4 (*Merck KGaA, Darmstadt, Germany*), sodium carbonate (99.5 %, *AppliChem, Darmstadt, Germany*), C7-C30 Saturated alkanes (1000μg/mL hexane, *Supelco Analytics, Bellefonte, PA, USA*), ultrapure water obtained by Millipore purification system (*Billerica, MA, USA*), ethanol (99.5%) (*VWR Chemicals, Fontenay-sous-Bois, France*), industrial washed sand (*VWR Chemicals, Leuven, Belgium*).

2.3. Determination of chemical composition

2.3.1. Determination of nitrogen content by Kjeldhal method

Two samples of each plant materials (*G. macrorrhizum* leaves 1,02 g, 1,02 g, roots 1,04 g, 1,03 g; *R. carthamoides* roots 1,04 g, 1,03 g) of 0,2 mm fraction were weighted to the special Kjeldahl flask. The substance (material) was heated in the flasks with 20 ml conc. entrated H_2SO_4 and the tablet of catalyst (K_2SO_3 3.4 g, $CuSO_4$ 0,4 g) for 2 hours until it became transparent. Heating intensity was 60%. Then the solution was distillated using automatic steam distillation system under the following conditions – 3 s NaOH, 3 s H_3BO_4, the time of distillation was 300 min, the intensity of the steam was 80 %. After distillation, the solution was collected into the flask, followed with the addition of Toshiro indicator and titration with 0,1 N HCl solution until the colour changes from light green to violet. 20 ml conc H_2SO_4 was used as a control sample and analyzed in the same manner which was described above. The nitrogen content was calculated using the following ***Equation 1*** (with expression in a percentage):

$$\%N = (V_A - V_B) * N_{HCl} * 1{,}4007/W; \text{ (1)}$$

where: V_A – volume of standard HCl required for the sample, ml; V_B - volume of standard HCl required for the blank, ml; N_{HCl} – normality of acid standard; 1,4007 – miliequivalent weight of N*100; W – weight of the sample, g.

2.3.2. Determination of oil content by Sohxlet-He extraction

Grinded *G. macrorrhizum* leaves and roots, *R. carthamoides* roots in a fraction of 0,2 mm and weight of 5±0.01 g were placed in cellulose extraction thimbles. Soxhlet extraction was performed in automated Soxhlet extractor EZ100H (Behr Labor-Technk, Düsseldorf, Germany). Cellulose extraction thimbles were inserted into an inner tube of Soxhlet extraction apparatus for the extraction in standard method which is described in Official Methods and Analysis of AOAC

International [99]. All extractions were performed in triplicates, non-polar fraction was isolated using hexane solvent. Total extraction time was 360 min, extraction rate 1 cycle per 5 min, temperature was 80 °C. Hexane was evaporated in a Büchi V-850 Rotarvapor R-210 (Flawil, Switzerland). After the organic solvent evaporation, extracts were kept under a nitrogen flow for 15 min to evaporate residues of hexane. Extract yields were determined gravimetrically (±0.001 g) and expressed as a % of dry weight.

2.3.3. Determination of water content

Grinded *G. macrorrhizum* leaves and roots, *R. carthamoides* roots in a fraction of 0,2 mm and weight of 5±0.03 g were placed in a dry, constant weight glasses with a cap and rod. Experiments were performed in duplicates. Glasses with a cap, rod and samples were weighted gravimetrically (±0.001 g), periodically stirred and dried in the oven at the 100-105 °C temperature. After every 24 h samples were cooled in the desiccator for 30 minutes and then weighted gravimetrically (±0.001 g) until the constant weight. The weight reached a plateau (the difference between the samples was 0.001-0.007 g) after 168 h (7 days). Water content was calculated using the ***Equation 2*** below and expressed in a g/100 g of dry weight (DW)

$$\boldsymbol{x} = \frac{(\boldsymbol{m_1}-\boldsymbol{m_2})\times\boldsymbol{100}}{\boldsymbol{m_1}-\boldsymbol{m}}; \text{ g/100 g of DW; (2)}$$

where: m – weight of the glass with a cap and rod, g; m_1 – weight of the glass with a cap, rod and the sample before drying, g; m_2 – weight of the glass with a cap, rod and the sample after drying, g.

2.3.4. Determination of ash content

Grinded *G. macrorrhizum* leaves and roots, *R. carthamoides* roots in a fraction of 0,2 mm and weight of 2±0.1 g were placed in a dry, constant weight crucible. Experiments were performed in duplicates. Crucibles with grinded samples were place on the electric hotplate and heated until the smoke stopped to form. After this, crucibles with samples were transferred and kept in the muffle under 600-650 °C temperature. After 16 hours weight stopped decreasing, reached a plateau (the difference between the samples was 0.001-0.005 g). Ash (mineral) content was calculated using the ***Equation 3*** below and expressed in a g/100 g of dry weight (DW):

$$\boldsymbol{x} = \frac{(\boldsymbol{m_1}-\boldsymbol{m_2})\times\boldsymbol{100}}{\boldsymbol{m_1}-\boldsymbol{m}}; \text{ g/100 g of DW; (3)}$$

where: m – weight of the crucible, g; m_1 – weight of the crucible with the sample before drying, g; m_2 – weight of the crucible with the sample after drying, g.

2.4. Isolation of bioactive *G. macrorrhizum* and *R. carthamoides* compounds by high-pressure extraction techniques

2.4.1. Analytical scale super critical CO_2 extraction (SFE-CO_2)

Super critical CO_2 extraction was performed in a supercritical fluid extractor Helix extraction system (Applied Separation, Allentown, PA, USA). During the experiment the volume of CO_2 was measured by ball-float rotameter and digital mass flow meter in a standard liter per minute (SL/ml) at standard parameters: P_{CO2}=100 kPa, ρ=0.0018 g/ml, T_{CO2}=20 °C. For each extraction grinded *G. macrorrhizum* leaves in a fraction of 0,2 mm and weight of 10±0.01 g were placed in a dry stainless steel extraction vessel (50 cm^3, 14 mm inner diameter, 320 mm length). On the top and bottom of extractor vessel absorbent cotton was placed to avoid release of the particles of material into the system. The static extraction took 10 minutes, dynamic extraction took from 15 min to 75 min, temperature range was from 40 °C to 60 °C, pressure from 200 bars to 500 bars and the CO_2 flow rate 2 ± 0.2 SL/min. Extracts were stored at -20 °C temperature and protected from the light until further analysis.

2.4.2. Experimental design

In order to determine the optimum conditions of super critical carbon dioxide extraction, three independent factors - pressure (200-500 bars), temperature (40-60 °C), time (15-75 min) - were selected using a rotatable central composite experimental design (CCD) with three levels for each factor. Super critical CO_2 extraction was optimized using response surface methodology (RSM) on the extraction yield (%). A total of 20 runs was performed in CCD. Extractions at 350 bars, 50 °C, 45 min (middle point) were performed in sextuplicates, the rest extractions were performed in dublicates. The order of experiments was randomized. Data were analyzed using response surface methodology with the Design expert Version 8 software (Stat-ease, Inc, Minneapolis, Minnesota, United States).

2.4.3. Pilot scale super critical CO_2 extraction (SFE-CO_2)

Super critical CO_2 extraction was performed in a supercritical fluid medium and a big PS extractor Helix extraction system (Applied Separation, Allentown, PA, USA). *G. macrorrhizum* leaves extraction was performed in a medium pilot scale extractor vessel (dimensions) with100 g of material extracted under 462 bars of pressure, temperature of 55 °C for 52 min (until it reached stable plateau). *G. macrorrhizum* leaves, roots and *R. carthamoides* roots extractions were

performed in a medium and large PS extractor (dimensions) with 100 g of material (medium PS), 1980 g (large PS), *G. macrorrhizum* roots weigth of 3750 g and *R. carthamoides* roots weight of 3520 g. under the same pressure and temperature for 420 min.

2.5. *In vitro* antioxidant activity

2.5.1. Measurement of total phenols content (Folin-Ciocalteu method)

Total phenols content (TPC) of the SFE-CO_2 extracts of *G. macrorrhizum* leaves, roots and *R. carthamoides roots* were determined by using Folin-Ciocalteu assay of Singleton, Orthofer & Lamueal-Raventós [100] with some modifications. The working solution was prepared by diluting commercial Folin-Ciocalteu's reagent in distilled water (1:9, v/v). 750 µL diluted Folin-Ciocalteu's solution was mixed with 150 µL of the sample (200-2000 µg/mL). MeOH was used for the blank. After 3 minutes, 600µL of Na_2CO_3 (75g/L) was added in to solution, left in the dark for 120 min at 25 °C.

QUENCHER. Total phenols content (TPC) of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots initial plant material and SFE-CO2 residues was measured by Pastoriza *et al* [101] method by directly applying it to the solid particles. Since all the samples had high antioxidant activity, they were diluted with inert material – microcrystalline cellulose. 750 µL diluted Folin-Ciocalteu's solution was mixed with 10 mg of sample and 150 µL distilled water in a test tubes. After 3 minutes, 600 µL of Na_2CO_3 (75g/L) was added to neutralized the mixture, vortexed in the dark for 120 min at 25 °C, centrifuged at 4500 rpm 5 min.

Absorbance was measured at 760 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY, USA).

Gallic acid solutions were used to obtain the calibration curve in the 0.006 to 0.2 mg/mL concentration range. Extracts (***Equation 4***) and QUENCHER (***Equation 5***) calibration curves equitations:

$$f\ (x) = 0.0140\ x + 0.0185;\ R^2 = 0.999; (4)$$

$$f\ (x) = 0.0143\ x - 0.0098;\ R^2 = 0.997; (5)$$

Total phenols content (TPC) was expressed in a mg of gallic acid equivalents (GAE) per g of an extract or plant material. All analysis was performed in quadruplicates.

2.5.2. **$ABTS^{\bullet+}$ cation radical assay**

The Trolox equivalents antioxidant capacity (TEAC) was measured by Re at al. [102] method with some modifications. The phosphate buffered saline (PBS; 75 mmol/L, pH 7.4) was prepared by dissolving 8.18 g NaCl, 1.42 g Na_2HPO_4, 0.27 g KH_2PO_4, 0.15 g KCl in 1 L distilled water. The $ABTS^{\bullet+}$ radical solution was prepared by reacting 50 mL of $ABTS^{\bullet+}$ (2 mmol/L PBS) and 200 μL $K_2S_2O_8$ (70 mmol/L) and left for 15-16 hours in the dark at at 25 °C before use. Then, the working solution was prepared by diluting $ABTS^{\bullet+}$ radical solution with PBS to obtain 0.700 (±0.01) AU at 734 nm. 1500 μL of $ABTS^{\bullet+}$ solution was mixed with 25 μL of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts (in a concentration range from 0.75 to 3 mg/mg) or MeOH (blank) in an Eppendorf vial and left for 2 hours in the dark at 25 °C.

QUENCHER. For *G. macrorrhizum* leaves, roots and *R. carthamoides* roots initial plant material and SFE-CO2 residues quencher analysis, 10 mg of sample (in a concentration range from 0,0005 to 0,005 mg/mg) or cellulose (blank) was mixed with 1500 μL of $ABTS^{\bullet+}$ solution and 25 μL MeOH, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark at 25 °C, centrifuged at 4500 rpm 5 min. Absorbance was measured at 734 nm with Spectronic Genesys 8 spetrophotometer (Thermo Spectronic, Rochester, NY). Trolox solutions (25 μL) at various concentrations (0-1500 μmol/l) were used to obtain the calibration curve. Extracts (***Equation 6***) and QUENCHER (***Equation 7***) calibration curves equitations:

$$f\ (x) = 0.0638\ x + 1.3042;\ R^2 = 0.999 \quad (6)$$

$$f\ (x) = 0.0625\ x - 2.804;\ R^2 = 0.997 \quad (7)$$

$TEAC_{ABTS}$ of samples was calculated by means of dose-response curves for Trolox. Results were expressed as TEAC values in a mg of Trolox per g of an extract or plant material. All analysis was performed in quadruplicates.

2.5.3. **$DPPH^{\bullet}$ radical scavenging assay**

$DPPH^{\bullet}$ radical scavenging assay was performed by Brand-Williams, *et al*., (1995) method [103] with some modifications. Working solution was prepared by mixing 1000 μL $DPPH^{\bullet}$ methanolic solution (~ 89.7 μmol/L, final absorbtion 0.800 ± 0.1 AU at 517 nm) and 500μL of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts (in a concentration range from 0.25 to 1 mg/mg) or MeOH (blank). The mixtures were keep in for 2 hours in the dark at 25 °C.

QUENCHER. For *G. macrorrhizum* leaves, roots and *R. carthamoides* roots initial plant material and SFE-CO2 residues quencher analysis 10 mg of sample (in a concentration range from 0,0005 to 0,002 mg/mg) or cellulose (blank) was mixed with 500 μL MeOH and 1000 μL 89.7 μmol/L DPPH$^{\bullet}$ methanolic solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark at 25 °C, centrifuged at 4500 rpm 5 min. Absorbance of all the samples was measured at 517 nm with Spectronic Genesys 8 spetrophotometer (Thermo Spectronic, Rochester, NY, USA). Trolox solutions (500μL) in various concentrations (0-50μmol/L MeOH) were used to obtain the calibration curve. $TEAC_{DPPH}$ of samples was calculated by means of dose-response curves for Trolox. Results were expressed as TEAC values in a mg of Trolox per g of a extract or plant material. All analysis was performed in quadruplicates.

2.5.4. Oxygen radical absorbance capacity assay (ORAC)

Oxygen radical absorbance capacity assay was performed by Prior *et al*. [104] method with slight modifications. *G. macrorrhizum* leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts was dissolved in MeOH. In a 96-well black opaque microplate with transparent flat-bottom, 25 μL of the sample or MeOH (blank) was mixed with 150 μL fluorescein solution (14 μmol/L). Then, microplates were sealed and preincubated for 15 min at 37 ºC. Concentration of *Geranium machrorrhizum* leaves extract was in the range from 0.3 to 0.5 mg/ml. Concentration of *Geranium machrorrhizum* roots extract was in the range from 0.75 to 1 mg/ml. Concentration of *R. carthamoides* extract was in the range from 0.3 to 0.5 mg/ml.

QUENCHER. For *G. macrorrhizum* leaves, roots and *R. carthamoides* roots initial plant material and SFE-CO_2 residues quencher analysis 10 mg of sample or cellulose (blank) was mixed with 150 μL PBS solution (75 mmol/L) and 900 μL fluorescein solution (14 μmol/L PBS), vortexed for 15 s, shaken at 250 rpm for 1 hour in the dark at 25 °C, centrifuged at 4500 rpm for 5 min. 175 μL of optically clear samples were transferred to the 96-well black opaque microplate with transparent flat-bottom, preincubated for 15 min at 37 ºC. Concentration of *Geranium machrorrhizum* leaves and roots grounded material was in the range from 0.003 to 0.004 mg/ml. Concentration of *R. carthamoides* grounded material was in the range from 0.005 to 0.01 mg/ml. After preincubation, with a multichannel pipette 25 μL of AAPH solution as a peroxyl radical generator (240 mmol/L) was added manually. The microplate with samples (extracts and dry material) was immediately placed in the FLUOstar Omega fluorescent reader (BMG Labtech, Offenburg, Germany). It was automatically shaken before each reading.

Fluorescein was measured (excitation wavelength 485 nm; emission wavelength 510 nm) each cycle (1 min x 1.1), total 120 cycles for SFE-CO_2 extracts and 150 cycles for dry plant

samples. Raw data was done by Mars software (BMG Labtech GmbH, Offenburg, Germany) and exported Excel 2003 (Microsoft, Roselle, IL). Antioxidants curves (fluorescence VS time) were normalized and from normalized curves, the area under the curve (AUC) was calcuted using ***Equation 8*** below:

$AUC = 1 + \sum_{i=1}^{i=150} \frac{f_i}{f_0}$ (8);

where: $f_0$is the initial fluorescence reading at 0 min, f_i is the fluorescence reading at time i.

Trolox solutions (150 μL) were used to obtain the calibration curve in the 0-500 μmol/L concentration range. Extracts (***Equation 9***) and QUENCHER (***Equation 10***) calibration curves equitations:

$f\ (x) = 0.1394\ x + 0.7395;\ R^2 = 0.993$ (9)

$f\ (x) = 0.1302\ x - 2.7856;\ R^2 = 0.987$ (10)

$TEAC_{ORAC}$ of samples was calculated by means of dose-response curves for Trolox. Results were expressed as TEAC values in mg of Trolox per g of an extract or plant material. All analysis was performed in quadruplicates. Fluorescein and AAPH solutions were prepared daily and used as a fresh.

2.6. Chemical characterization of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots

2.6.1. Non-volatile compounds analysis by UPLC/ESI-QTOF-MS

Phytochemical composition of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts (in concentration1 mg/mg) was analysed as previously described by Kraujalis and Venskutonis [105] by UPLC-ESI-Q-TOF-MS with Acquity UPLC system (Waters, Milford, MA, USA) combined with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), binary solvent delivery system, an auto sampler with a 10 μL loop of all samples, column manager, photodiode array (PDA) detector a data station running the Compass acquisition and data software. An Acquity BEH C18 column (1.7 m 50 x 2.1 mm, i.d.) was used to separate compounds at 25 °C. The column was equilibrated for 1 min before each analysis. The mobile phase was composed by eluent A and eluent B. Eluent A was 0.4 v/v formic acid in ultra-pure water followed by an increasing eluent B (acetonitrile) from 0 to 100 % over 9 min. During the following 2 min, the amount of eluent B was maintained at 100 for 1 min, followed by the re-introduced initial conditions over 1 min the equilibration time of 1 min. In the PDA detector the effluent was monitored at 254 nm and was directly introduced in to the UHR-TOF mass spectrometer equipped with an ESI source. During analysis the flow rate was 0.4 mL/min, injection

volume μL. Mass spectrometry data was in ESI negative ionization mode. Instrument was controlled, and data were collected by the Compass 1.3 (HyStar 3.2 SR2) software. Preliminary compounds analysis was carried out by comparing accurate masses of compounds with masses reported in literature sources and the chemical database Chemspider.

2.6.2. Volatile compounds analysis by GCxGC/TOF MS

SFE-CO_2 extracts of *G. macrorrhizum* leaves were analyzed by Two-Dimensional Gas Chromatography/Time-of-Flight Mass Spectrometry (GCxGC/TOF MS). Analyses were performed using LECO Pegasus 4D system, consisting of an Agilent 7890 GC hardware control system, a GERSTEL Multipurpose Sampler MPS (*Gerstel GmbH, Mulheim an der Ruhr, Germany*), TOF MS detector (LECO, St. Joseph, MI, USA). Volatile compounds were separated using primary BPX-5 column (29.9 M, 250 μm, 0,25 μm film thickness) (*SGE Analytical Science, Australia*) linked with a secondary column, BPX-50 (1.580 m, 100 μm i.d., 0.1 μm film thickness). Flow path was: GC oven - length 29.9 m, internal diameter 250 μm, maximum temperature 360 °C, film thickness 0.25 μm; Modulator length – 0.1 m, internal diameter 250 μm, maximum temperature 360 °C, film thickness 0.25 μm; Secondary column – 1.58 m, internal diameter 100 μm, maximum temperature 330 °C, film thickness 0.1 μm; Detector - Modulator length – 0.21 m, internal diameter 100 μm, maximum temperature 330 °C, film thickness 0.10 μm. Carrier gas was helium. Target flow was 1 mL/min. Front inlet septum purge flow 3 mL/min. Column front inlet purge time – 30 sec, flow 20 mL/min, the actual flow to the inlet during pre-run and during a run before purge time 11 mL/min.

The oven temperature programme was as follows: 50 °C (0.2 min) then ramped to 300 °C at 15 °C/min (for 5 min); the secondary oven programme was the following: 65 °C (0.2 min) then ramped to 295 °C at 15 °C/min (for 5 min). The transfer line temperature was 250 °C. The toal GC method time – 1312 s. The mass range used for identification was from 35 to 550 *m*/*z* units, the TOF MS acquisition rate was 10 spectra/sec. Detector voltage was set at 1550 V and ion source temperature of 250 °C. Data from the GC×GC-TOFMS system was collected by ChromaTOF software v.4.22 (LECO).

Tentative identification. The minimum similarity accepted was 800. Minimum molecular weight allowed was 33, maximum was 550. For tentative identification of compounds MS and RI methods have been used. MS: By comparing their mass spectra with those know components stored in the Adams, MainLib, and Replib mass spectral libraries. RI: by comparing obtained retention indexes with those reported from Adams and NIST. Unique mass was used for the area calculation. The relative percentage of the chemical compounds of SFE-CO_2 extracts from leaves

of G. *macrorrhizum* was expressed as percentage by peak area. The quantity of sesquiterpene Germacrone was identified by comparing retention times and mass spectra with the standard (Geramcrone, ≥99.0%) solution. The content of this sesquiterpene was calculating according to the calibration curve. Calibration curve was drawn by plotting Germacrone peak area versus their concentrations added in the solution (from 0.01 to 0.1 mg/ml). Each point was done of three replicates. The regression curve equation (y = ax +b) obtained for Germacrone with the corresponding regression coefficient R^2 (***Equation 11)***:

$$y = 798{,}531{,}096.00 * x - 1{,}919{,}008.99,\ R^2 = 1.00 \quad (11)$$

2.7. Conventional extraction techniques

Solid-liquid extraction (SLE)

Solid-liquid extractions with different solvents (hexane, acetone, ethanol) were performed in a thermostatically controlled shaker. *G. macrorrhizum* leaves, roots and *R. carthamoides* roots in a fraction of 0,2 mm and the weigth of 10 g were loaded into dry glass bottles. 100 ml of different solvents (hexane, acetone, ethanol) was poured on the different grinded plants (*G. macrorrhizum* leaves, roots, *R. carthamoides* roots). Glass bottles with solvents and grinded samples were shaken at 800 rpm. All extractions were performed in duplicates. Every extraction took 360 min and was performed at 20 °C. After extraction, it rapidly cooled down, centrifugated (9000 rpm, 10 min) and then filtered (Whatman filter paper 1). All the organic solvents were evaporated in a Büchi V-850 Rotarvapor R-210 (Flawil, Switzerland). After organic solvent evaporation, extracts were kept under the nitrogen flow for 15 min to evaporate residues of organic solvents. All extracts were kept at -20 ºC protected from light until next analysis. SLE-He, SLE-ACN, SLE-EtOH extracts yields were determined gravimetrically (±0.001 g) and expressed in a % of dry weight.

2.8. Downstream valorization

Downstream valorization of *G. macrorrhizum* fat-free (SFE-CO_2 residues) leaves material which could be potentially coupled with bio-refinery process was performed in Foodomics Laboratory, Institute of Food Science Research (CIAL, CSIC), Nicolas Cabrera 9, 28049, Madrid Spain.

2.8.1. Pressurized liquid extraction (PLE)

Pressurized liquid extraction was applied to defatted *G. macrorrhizum* leaves residues after super critical carbon dioxide extraction. PLE was performed in a pressurized liquid extractor ASE 200 (Dionex, Sunnyvale, CA, USA) which was equipped with a solvent controller unit. For each extraction, dry stainless-steel extraction vessel (11 cm^3) was filled with 2 g of sea sand and cellulose filters in each ends. 0.5±0.0001 g of grinded SFE-CO_2 residue of *G. macrorrhizum* leaves in a fraction of 0.2 mm were mixed with 0.5±0.0001 g of sea sand and placed in the middle of vessel. Sea sand was used as a dispersive agent. Ultrapure water and ethanol were used as solvents. Before extractions, solvents were degassed with a sonicator for 10 min. Firstly, extraction cell was filled with extraction solvent (100 % of ethanol, mixture of 50% of ethanol and 50% water or 100% of water) and the pressure was increased to 1500 Psi. Then heat-up was applied depending on extraction temperature (50, 110, 170 ºC). Static extraction took 20 min. During static extraction all valves were closed to keep 1500 Psi constant pressure. Extract was collected in extraction vial and cell was rinsed with 60 % of extraction vial of the same selected solvent. In the end, all the extraction lines and cell were purged with nitrogen for 60 s. The PLE system was washed out between each extraction to avoid any carry-over from previous run. After pressurized liquid extraction, extracts were evaporated using nitrogen flow and freeze dryer (Labconco Corporation, Missouri, USA), depending on the used solvent. Extracts were stored at -20 °C temperature and protected from the light until further analysis.

2.8.2. Experimental design

In order to determine the optimum conditions of pressurized liquid extraction, two independent factors - percentage of ethanol in the solvent mixture (0-100%) (v/v) and temperature (50-170 ºC) - were selected using a rotatable central composite experimental design (CCD) with three levels for each variable. Thus, pressurized liquid extraction was optimized using a 3-level factorial design 3^2 studying the effect on extraction yield, total phenolic content (mg GAE/g extract), $DPPH^{\bullet}$ radical scavenging (EC_{50}, µg/ml) and antioxidant capacity (mM Trolox/g extract). A total of 20 experiments were analyzed including two center points (50% of ethanol, 110 ºC). The order of the experiments has been fully randomized for the protection against the effect of lurking variables. Data were analyzed using response surface methodology with the Statgraphics Centurion XVI software (Stat-Point Technologies, Inc., Warrenton, VA, United States). The effect of the independent variables on the response values were analyzed using pure error, considering a level of confidence of 95% for all the runs. Response optimization was carried out by the combination of experimental factors (pressure, temperature, time), looking for maximizing the

response (yield, %). The quadratic model proposed for each response variable can be expressed through the following ***Equation 12***:

$$Y_i = \beta_0 + \beta_1 T + \beta_2 E + \beta_{1,1} T^2 + \beta_{2,2} E^2 + \beta_{1,2} T * E + error, \quad (12)$$

where: T is temperature, E is percentage of ethanol in the solvent mixture, β_0 is intercept, β_1 and β_2 are linear coefficients, $\beta_{1,1}$ is the coefficient of two factors interaction, error is an error variable.

All models were evaluated considering the percent variation explained by the residual standard deviation (RSD), determination coefficient (R^2) and lack-of-fit test for the model from the analysis of variance table, as the significance criteria. Standardized Pareto chart was used to analyze the effect of each factor and its statistical significance for each response values at 99% confidence level. Response surfaces were obtained by accepting significances at $p \leq 0.05$. By the combination of two experimental factors, looking to maximize yield, total phenolic content, antioxidant activity and $DPPH^{\bullet}$ radical scavenging, multiple response optimization was carried out.

2.8.3. Measurement of total phenols content (Folin-Ciocalteu method)

Total phenols content (TPC) of the pressurized liquid extraction products of defatted *G. macrorrhizum* leaves (SFE-CO_2 plant residues) were determined spectrophotometrically by using Folin-Ciocalteu assay of Montero *et al*. [106]. Procedure was miniaturized with 1 mL of reaction. Briefly, 10 µL of sample (0.625 mg/ml) was mixed with 600 µL ultra-pure water. Then 50 µL of undiluted Folin-Ciocalteu reagent was added. After shaking in a vortex and waiting 1 min, 150 µL of 20 % (w/v) Na_2CO_3 was added. Then, 190 µL of ultra-pure water was added to complete the volume of 1 mL. Samples were incubated for 2 h at room temperature (25 ºC) in the darkness. 300 µL of each mixture was transferred to 96-well microplate. Experiment was done in triplicate. Absorbance was measured at 760 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). Solvent in which the sample is dissolved (100 % of ethanol, mixture of 50% of ethanol and 50% water or 100% of water) was used as blank. Serial gallic acid solutions (0.031-2 mg/ml) were used to obtain the calibration curve (***Equation 13)***:

$$f(x) = 0.933\,x + 0.0273;\ R^2 = 0.998; \quad (13)$$

Total phenols content (TPC) was expressed in a mg of gallic acid equivalents (GAE) per g of an extract.

2.8.4. DPPH$^{\bullet}$ radical scavenging assay

DPPH$^{\bullet}$ radical scavenging assay was performed by Brand-Williams, *et al*., (1995) method [103] with some modifications. A stock solution was prepared dissolving 23.5 mg of DPPH$^{\bullet}$ 100 µL methanol. Working solution was prepared by diluted stock solution to 1:10 with methanol. Both solutions were stored at 4 ºC until further experiments. 290 µL of DPPH$^{\bullet}$ diluted solution was mixed with 10 µL of sample (5 different concentration in a range from 0.0391 to 0.6250 mg/ml) in an Eppendorf vial and left for 4 hours in the dark at 25 °C. 300 µL of each mixture was transferred to 96-well microplate. Experiment was done in triplicate. Absorbance was measured at 516 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). DPPH$^{\bullet}$ methanolic solutions were used as a control sample. The reaction medium of remaining DPPH$^{\bullet}$ concentration was calculated from a calibration curve. The percentage of remaining DPPH$^{\bullet}$ against the extract was plotted to obtain EC_{50} (the amount of antioxidant necessary to decrease the initial DPPH$^{\bullet}$ concentration by 50%).

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

The Trolox equivalents antioxidant capacity (TEAC) was measured by Re at al. [102] method with some modifications. The phosphate buffered saline (PBS; 50 mM, pH 7.4) was prepared by dissolving 1.7011g of KH_2PO_4 in 250 ml (Solution A) and 7.0990 Na_2HPO_4 in 1000 ml (Solution B) of ultra-pure water. Then, mixing 190 ml of Solution A with 810 ml of Solution B. 7 mM ABTS$^{\bullet+}$ solution was prepared by dissolving 0.0096 g of ABTS$^{\bullet+}$ to 2.5 ml ultra-ure water. 139.8 mM potassium persulfate solution was prepared by dissolving 0.0378 of potassium persulfate in 1 ml of water. These solutions were prepared fresh each day of analysis. ABTS$^{\bullet+}$ radical was prepared by mixing 7 mM of ABTS$^{\bullet+}$ and 2.45 mM of potassium persulfate. Radical was left for 16 hours in the dark at 25 °C before use. After reaction of ABTS$^{\bullet+}$ radical was completed, 1 ml of ABTS$^{\bullet+}$ radical was diluted with ~70 ml of 5 mM PBS (pH 7.4) until absorbance of 0.700 (±0.02) AU at 734 nm was obtained. 1000 µL of ABTS$^{\bullet+}$ solution was mixed with 10 µL of sample (5 different concentration in a range from 0.0391 to 0.6250 mg/ml) in an Eppendorf vial and left for 4 hours in the dark at 25 °C. 300 µL of each mixture was transferred to 96-well microplate. Experiment was done in triplicate. Absorbance was measured at 734 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments, Winooski, VT, USA). Trolox solutions were used as reference standards. Results were expressed as TEAC values (mmol of trolox/g extract). These values were obtained from five different concentrations of each extract

that were tested in the assay. Values in the linear response between 20 and 80 % of blank absorbance were used in the analysis only.

2.8.6. Analysis of the phytochemical profile of *G. macrorrhizum* PLE extracts by Liquid chromatography-mass spectrometry (UPLC/ESI-QTOF-MS/MS)

The polyphenolic profile of Geranium extracts was determined using an ultrahigh performance liquid chromatography (UPLC) system 1290 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of-flight mass spectrometer (q-TOF MS) Agilent 6540 that was equipped with an orthogonal ESI source (Agilent Jet Stream, AJS, Santa Clara, CA, USA), and controlled by a PC running the Mass Hunter Workstation software 4.0 (MH) from Agilent. A chromatographic method was carried out using a Zorbax Eclipse Plus C18 column (2.1 × 100mm, 1.8 μm particle diameter, Agilent Technologies, Santa Clara, CA) at 30 °C. Mobile phase composition was water (+0.1% formic acid, A) and acetonitrile (+0.1% formic acid, A). This method was developed to establishing phytochemical profiling. The gradient program was as follows: 0 min, 0% B; 12 min, 80% B; 14 min, 100% B; 16 min, 100% B; 17 min, 0% B. A flow rate of 0.5 mL/min and an injection volume of 5 μL were employed. The analysis was performed in negative ion mode (ESI-) for second method. The mass spectrometer was used in MS and MS/MS modes for the structural analysis of all compounds. MS parameters were the following: capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow rate, 10 L/min; gas temperature, 350 ºC; skimmer voltage, 45 V; fragmentor voltage, 110 V. The MS and Auto MS/MS modes were set to acquire m/z ranging between 50-1100 and 50-800 amu, respectively, at a scan rate of 5 spectra per second. Extracts were dissolved at a concentration of 50 mg/mL in ethanol prior to liquid chromatography analysis.

2.9. Statistical analysis

Microsoft Excel 2016 was used for calculation mean values and standard deviations. GraphPad Prism 6.01 software was used for one-way analysis of variance (ANOVA), followed by the Tukey's test in order to compare mean values and their significance (p-value < 0.05).

3. RESULTS AND DISCUSSION

3.1. Chemical composition

In the first step of this study chemical composition of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots was characterized. Results are presented in ***Table 3.1.*** The nitrogen content of all the samples ranged between 0.56 % and 2.66 %. *G. macrorrhizum* leaves had the highest nitrogen content (2.66%, ***Table 3.1***). The value of *G. macrorrhizum* roots *and R. carthamoides* roots was similar (0.89, 0.56 %, respectively). The fat content of all the samples ranged between 0.67 % and 3.97 %. Determination of fat content by Soxhlet-He extraction technique shows that fat content of *G. macrorrhizum* leaves are almost 2 times higher than in *G. macrorrhizum* roots and six times higher than in the *R. carthamoides* roots (3.97, 1.91, 0.67 %, respectively). Graça *et al*. have also reported the fat content of *Geranium* plant *(Geranium molle L)* and their results was 15.50 %. According to the same author, another genus *Geranium robertianum L* has 15.60 % content of fat [107]. *R. carthamoides* roots showed the highest ash content (15.64%). It is three times higher than in *G. macrorrhizum* roots (4.97 %) and two times higher than in *G. macrorrhizum* leaves (7.82 %). On Graça *et al*. report, the ash content of *Geranium molle L* [108] and *Geranium robertianum L.* [107] was 10.50 and 9.80 %, respectively. Meanwhile, the water content of all the samples ranged between 6.80 % and 12.00 %. *G. macrorrhizum* roots had the highest water content (12.00%) among all the studied plants. *R. carthamoides* roots showed the lowest water content (0.67 %). It is two times lower than in *G. macrorrhizum* roots (12.00 %). The water content of both *G. macrorrhizum* samples (leaves and roots) obtained in this study has been much lower compared to the previous study. Graça *et al*. analysed water content of *Geranium molle L* specimens in blossom [108] and *Geranium robertianum L.* [107], it was 72.20 and 84.40 %, respectively. To the best of my knowledge there are no previous reports on *G. macrorrhizum* and *R. carhtamoides* chemical composition.

Table 3.1. Chemical composition of *G. macrorrhizum* leaves and roots, *R. carthamoides* roots.

	G. macrorrhizum		***R. carthamoides***
	Leaves	***Roots***	***Roots***
Kjeldahl, nitrogen content, %	2.66 ± 0.01^{c}	0.89 ± 0.02^{b}	0.56 ± 0.00^{a}
Soxhlet-He extraction yield, %	3.97 ± 0.03^{c}	1.91 ± 0.02^{b}	0.67 ± 0.04^{a}
Ash content, %	7.82 ± 0.53^{b}	4.97 ± 0.19^{a}	15.64 ± 0.50^{c}
Water content, %	10.30 ± 0.00^{b}	12.00 ± 0.11^{c}	6.80 ± 0.50^{a}

Values represented as mean ± standard deviation. Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, Tukey's test, $p < 0,05$).

3.2. Supercritical carbon dioxide extraction of *G. macrorrhizum* and *R. carthamoides*

Optimisation of SFE-CO_2 parameters

Supercritical carbon dioxide extraction (SFE-CO_2) was applied for *Geranium machrorrhizum* leaves, roots and *Rhaponticum cahhtamodes* roots to obtain bioactive SFE-CO_2 extracts.

Due to their high fat content (3.97 %, ***Table 3.1.***) *G. macrorrhizum* leaves were selected for the optimization of SFE-CO_2. Response surface methodology (RSM) was used to optimize three independent variables (pressure, extraction time and temperature) for the yield maximization. Thus, optimum extraction conditions were determined using three independent factors pressure (200-500 bars), temperature (40-60 °C), time (15-75 min). RSM methodology frequently has been used for extraction experiments optimization [109], [110].

Table 3.2 presents the yields of SFE-CO_2 extracts extracted under different conditions. Extraction yield was expressed in percentage of dry mass. Obtained yield ranged from 1.69 to 3.29 %. The maximum yield (3.29 %) was obtained under 500 bars, 60 °C and 75 min.

Overall, the optimum conditions - pressure, temperature and time for SFE-CO_2 extraction in this study were found to be 462 bars for pressure, 55 °C for temperature and 52 min for extraction time. To determine the validity of predictive model, predicted and experimental values were compared (***Table 3.3***). The actual optimal SFE-CO_2 extracts yield value (3.35 %) well fitted the predicted optimal yield value (3.23 %). Predicted and actual SFE-CO_2 extraction yields are also presented in ***Figure 3.1***. The dots of the predicted and actual values are close to the 45° line, thus it shows a normal distribution and confirms that the model is well adapted.

The regression equation which has been fitted to the model (***Equation 14***):

$$Extraction\ yield, \% = 1.03966 - 0.000371656 * P + 0.00710017 * T + 0.0395836 * t - 0.00000303145 * P^2 + 0.000109806 * P * T - 0.0000274631 * P * t - 0.000244577 * T^2 - 0.0000884708 * T * t - 0.000213842 * t^2, \quad (14)$$

where: P is pressure, T is temperature, t is time

Table 3.2 Super critical carbon dioxide extraction on analytical scale parameters and extraction yields

SFE-CO_2 parameters			**Extraction yield, %**	
Pressure, bar	*Temperature, °C*	*Time, min*	*Actual*	*Predicted**
350	50	45	2,97 ± 0,16	*2,92*
200	60	75	2,63 ± 0,15	*2,66*
200	40	75	2,69 ± 0,24	*2,70*
500	50	45	3,15 ± 0,11	*3,11*

Table 3.2 Super critical carbon dioxide extraction on analytical scale parameters and extraction yields

350	50	45	2,88 ± 0,16	*2,92*
200	50	45	2,66 ± 0,08	*2,59*
200	40	15	1,98 ± 0,18	*2,03*
350	50	45	2,89 ± 0,16	*2,92*
350	60	45	3,08 ± 0,06	*3,07*
200	60	15	2,11 ± 0,15	*2,09*
500	60	75	3,29 ± 0,04	*3,28*
350	50	15	2,56 ± 0,05	*2,54*
350	50	75	2,99 ± 0,03	*2,92*
350	50	45	2,93 ± 0,16	*2,92*
500	40	15	2,48 ± 0,02	*2,47*
350	50	45	2,79 ± 0,16	*2,92*
500	40	75	2,61 ± 0,04	*2,66*
500	60	15	3,18 ± 0,06	*3,20*
350	50	45	2,89 ± 0,16	*2,92*
350	40	45	2,82 ± 0,08	*2,73*
Optimal conditions:				
462	55	52	3,35±0,09	*3,23*

Values represented as mean ± standard deviation. *Predicted values were suggested by Design-Expert 7.0.0 software Yield Analysis.

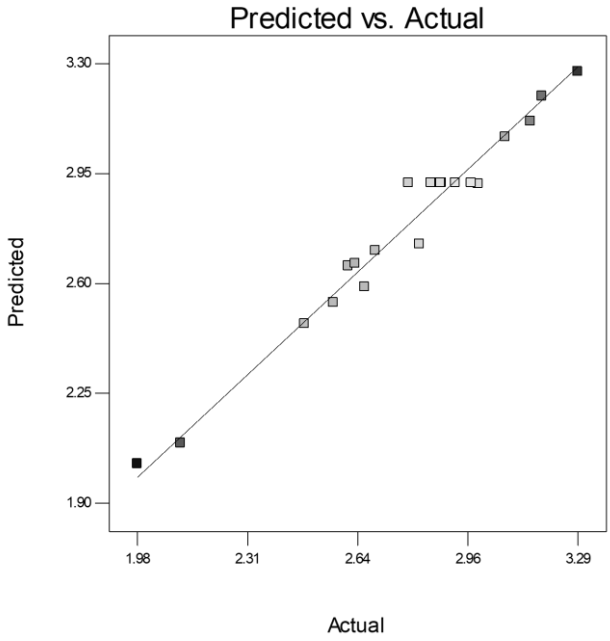

Figure 3.1. Predicted and actual SFE-CO2 extraction yields.

The ANOVA table (***Table 3.3***) partitions the variability of the extraction yield into separate pieces for each of the factors. It tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 7 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence

level (model, pressure, temperature, time, interaction between temperature and pressure, interaction between time and pressure, quadratic effect of time). The SFE-CO_2 extraction yield was not significantly affected by interaction between the time and temperature, as can be seen in ***Table 3.3.***

The R-Squared statistic indicates that the model as fitted explains 97.34% of the variability in extraction yield. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, was 94.94%.

Table 3.3. Analysis of Variance (ANOVA) for extracton yield

Source	**SS**	**df**	**MS**	**F-Ratio**	***p*-value**
Model	2,02	9	0,22	40,60	< 0,0001*
A-Pressure	0,70	1	0,70	126,16	< 0,0001*
B-Temperature	0,29	1	0,29	52,84	< 0,0001*
C-Time	0,36	1	0,36	64,65	< 0,0001*
AB	0,22	1	0,22	39,25	< 0,0001*
AC	0,12	1	0,12	22,10	0,0008*
BC	*5.636E-003*	1	*5.636E-003*	1,02	0,3365
A^2	*0,013*	1	*0,013*	2,31	0,1592
B^2	*1.645E-003*	1	*1.645E-003*	0,30	0,5974
C^2	*0,10*	1	*0,10*	18,42	0,0016*
Residual	0,055	10	5.529E-003		
Lack of fit	0,035	5	*7.059E-003*	1,77	0,2740
Pure error	0,020	5	*3.999E-003*		
Total SS	2,08	19			

*Significant value ($p<0.05$).

The significances of pressure, time and temperature on extraction yield are shown on Pareto chart (***Figure 3.2)***. Extraction yield is mostly influenced by linear factors of pressure, time and temperature. 3D and 2D response surface plots are shown in ***Figure 3.3***. They are a graphical presentation of regression equations. Plots helped to better understand the interaction between variables and see the maximal response. Analysis of the generated response surfaces revealed that the highest responses were obtained using higher temperature and pressure. Regarding extraction time, the yield increased with increasing time up to ~50 min. However, further increases of extraction time did not result in improvements on the extraction yield.

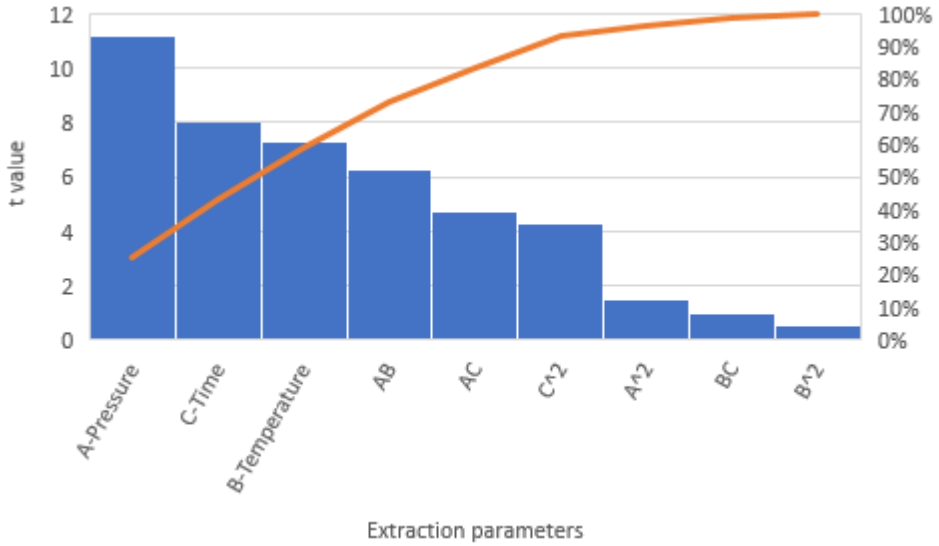

Figure 3.2. Pareto chart for the extraction yield response variables studied in the experimental design

The effect of temperature and pressure on extraction yield (τ = 45 min):

The effect of time and pressure on extraction yield (T = 50 °C):

The effect of time and temperature on extraction yield (P = 350 bar):

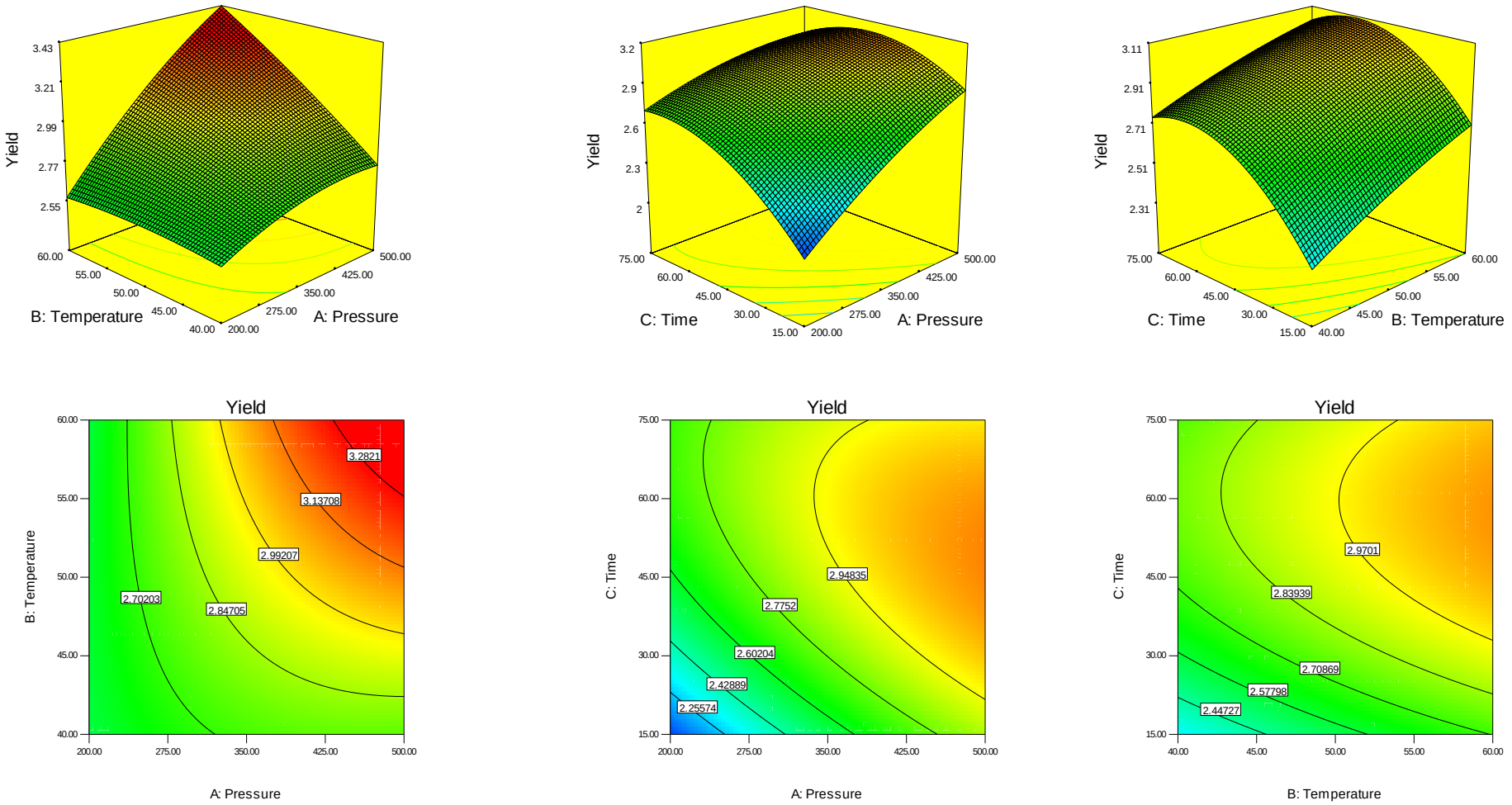

Figure 3.3. Response surface 3D and 2D plots.

Optimal conditions (temperature and pressure) of *G. macrorrhizum* leaves on analytical scale super critical CO_2 extraction were used for *G. macrorrhizum* leaves, roots and *R. carthamoides* roots on pilot scale SFE-CO_2 extraction (***Table 3.4***). The SFE-CO_2 extracts yields under these conditions was 3.15 % (*G. macrorrhizum* leaves on medium pilot), 3.41 % (*G. macrorrhizum* leaves on large pilot), 1.2 % (*G. macrorrhizum* roots on large pilot) and 0.74 % (*R. carthamoides* roots on large pilot). SFE-CO_2 extracts yield of *G. macrorrhizum* leaves obtained by laboratory scale SFE-CO_2 (3.23 %) and SLE-He (2.92 %) was similar to pilot medium (3.15 %) and large scale extraction yield (3.41 %).

Table 3.4. Super critical carbon dioxide extraction on pilot scale parameters and yields

	Pilot SFE – CO_2 conditions			
	Pressure, bar	Temperature, °C	Time, min	Yield, %
G. macrorrhizum leaves^M	462	55	52	3,15±0,05
G. macrorrhizum leaves^L	462	55	420	3,41**
G. macrorrhizum roots^L	462	55	420	1,2**
R. carthamoides roots^L	462	55	420	0,74**

Values represented as mean ± standard deviation. *Predicted values were suggested by Design-Exprt Software Yield Analysis. **No repetition. ^{M}Medium pilot scale supercritical carbon dioxide extractor. ^{L}Large pilot scale supercritical carbon dioxide extractor

Optimum extraction conditions may vary depending on plant genus and variable of interest. Peterson *et al*. did studies on supercritical fluid (CO_2) extraction of *Pelargonium graveolens* (*Geraniaceae* family) [91]. 300 bars, 40 °C was the optimum conditions for extraction that gave 2.53 % extraction yield. Peterson reports that under these conditions the yield was not the highest, but wax content was lower than using lower pressure (100 bar) and higher temperature (70 °C). In this analysis the extraction yield under similar conditions (350 bars, 40 °C) was a bit higher (2.87 %). Under optimized extraction conditions the SFE-CO_2 extracts yield was higher, but the yield may be shifted because of the waxes. Here further investigation of chemical composition needs to be done. Gomes *et. al.* reported that supercritical carbon dioxide extraction yield of green leaves of rose geranium plant (*Pelargonium sp.)* varied between 0.019 and 0.22% [111]. Moreover, authors studied the effect of extraction conditions on the yield (pressure, temperature, time). Results showed that temperature and pressure are the most important operating parameters. Gomes *et al.* compared SFE-CO_2 and SLE-He extraction yield. SLE-He method showed higher extraction yield than SFE-CO_2 (0.35, 0.22 %, respectively).

3.3. *In vitro* antioxidant activity

G. macrorrhizum leaves, roots and *R. carthamoides* roots were examined for their *in vitro* antioxidant activity. The total phenolic content and antioxidant activity of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots are shown in ***Table 3.5.*** Total phenolic content of the plant material and extracts is expressed as milligrams of gallic acid equivalents per gram. The values of TPC ranged between 21.47 and 184.12 mg GAE/g DW of all initial plant materials, 0.21 and 2.66 mg GAE/g DW of SFE-CO_2 extracts, 23.01 and 130.23 mg GAE/g DW of SFE-CO_2 plant residues. The highest total phenolic content was obtained in *G. macrorrhizum* leaves initial plant material (184.12 mg GAE/g DW).

The values of $ABTS^{\bullet+}$ cation radical assay ranged between 110.53 and 1011.53 mg GAE/g DW of all initial plant materials, 0.56 and 12.25 mg GAE/g DW of SFE-CO_2 extracts, 320.64 and 741.62 mg GAE/g DW of SFE-CO_2 plant residues. In $ABTS^{\bullet+}$ cation radical assay, the highest antioxidant activity had *G. macrorrhizum* roots initial plant material (1011.53 mg TE/g DW).

The antioxidant activity of the extracts also was measured by the ability to scavenge DPPH• free radicals. This method is a sensitive way for determination of antioxidant activity [113]. The values of DPPH• cation radical assay ranged between 30.42 and 490.55 mg GAE/g DW of all initial plant materials, 0.11 and 0.86 mg GAE/g DW of SFE-CO_2 extracts, 89.15 and 496.67 mg GAE/g DW of SFE-CO_2 plant residues. DPPH• radical scavenging results showed the highest antioxidant activity of *G. macrorrhizum* roots SFE-CO_2 plant residue material (496.67 TEAC mg Trolox/g DW). In this case *G. macrorrhizum* roots initial material of roots has lower capacity to bind radicals (490.55 TEAC mg Trolox/g DW) than plant residues after supercritical carbon dioxide extraction. Although, the difference is small, antioxidant activity can be changed due to changes in the matrix. It was observed that plant material has higher scavenging activity than SFE-CO_2 extracts. Thus, only a small part of compounds with antioxidant activity are extracted from the plant material using SFE-CO_2 extraction technique the main part of bioactive constituents remains in the plant material.

The values of oxygen radical absorbance capacity assay ranged between 1.24 and 6.31 mg GAE/g DW of SFE-CO_2 extracts, 52.25 and 75.18 mg GAE/g of SFE-CO_2 plant residues. The highest oxygen radical absorbance capacity assay was obtained by *G. macrorrhizum* plant leaves residues after SFE-CO_2 extraction (75.18 TEAC mg Trolox/g DW).

The variations of scavenging activities of the initial plant materials could be attributed to prevailed antioxidant molecules such as phenols that are unequal in different parts of plants and material. Interestingly, the highest TPC values was demonstrated in *G. macrorrhizum* plant leaves and roots material as well as the highest $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ values, comparing with *R.*

carthamoides. Obtained TPC values and antioxidant activity are in a good agreement confirming that phenolic content is correlated with antioxidant activity. On the other hand, antioxidant activity also depends on compounds absorbtion mechanism and biotransfromation [114]. Thus, *in vivo* investigations are required.

Boukhris *et al*. [115] analyzed total phenolic content of polar extracts and SFE-CO_2 extracts of *P. graveolens* (*Geraniaceae* family). Authors obtained the highest total phenolic content in Sohxlet-methanol extracts of leaves (84.18 mg GAE/g) and flowers (109.76 mg GAE/g). Korcan *et. al.* reported total phenolic content of methanolic extract of *G. macrorrhizum* (76.33 mg GAE/g) [116]. Both values are similar to SFE-CO_2 extract of *G. macrorrhizum* leaves (79.38 mg GAE/g). Alali *et al*. reported TPC of the methanolic extract of *Erodium bryoniifolium* (*Geraniaceae* family), it was three times lower than *G. macrorrhizum* roots and eight times lower than *G. macrorrhizum* leaves SFE-CO_2 extract (10.8, 30.87, 79.38 mg GAE/g, respecively) [117]. The same author reported TPC content of methanolic extract of *Echinops philistaeus* (*Asteraceae* family), it was 19.2 mg GAE/g [117]. *R. carthamoides* initial plant material total phelic content was almost the same (19.64 mg GAE/g).

In the previous study of the extracts and essential oils of *Geraniaceae* family plants showed high antioxidant capacity [118], [119]. Antioxidant capacity determined by $ABTS^{\bullet+}$ method for the methanol extract of *P. quercetorum* was 668.4 mg TEAC/g and 293.3 mg TEAC/g for the water extract. Water extract antioxidant capacity was similar to SFE-CO_2 extract of *G. macrorrhizum* leaves capacity (365.74 TEAC mg TE/g).

Table 3.5. Total phenolic content and antioxidant activity of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots initial plant material, SFE-CO_2 extracts and SFE-CO_2 plant residue.

	G. macrorrhizum		***R. carthamoides***
	Leaves	***Roots***	***Roots***
TPC, mg GAE/g*			
SFE-CO_2 extract:			
mg/g extract	79,38±3,74[b]	30,87±2,69[a]	28,99±2,99[a]
mg /g DW	2,66±0,13 [c]	0,37±0,03 [b]	0,21±0,02 [a]
Plant material:			
Initial, mg/g DW	184,12±9,66[b]	176,25±9,18[b]	21,47±1,36[a]
Residues after SFE-CO_2	108,16±4,31[b]	130,23±5,37[c]	23,01±1,21[a]
$ABTS^{\bullet+}$, TEAC mg TE/g**			
SFE-CO_2 extract:			
mg/g extract	365,74±18,49	N/A	75,59±3,35
mg/g DW	12,25±0,62	N/A	0,56±0,02
Plant material:			
Initial, mg/g DW	980,21±76,99 [b]	1011,53±95,27 [b]	110,53±10,64 [a]
Residues after SFE-CO_2	741,62±47,42	N/A	320,64±8,36
DPPH•, TEAC mg TE/g**			
SFE-CO_2 extract:			

Table 3.5. Total phenolic content and antioxidant activity of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots initial plant material, SFE-CO_2 extracts and SFE-CO_2 plant residue.

mg/g extract	25,73±0,94 [c]	9,38±0,14 [a]	14,23±0,70 [b]
mg/g DW	0,86±0,03 [b]	0,11±0,00 [a]	0,11±0,01 [a]
Plant material:			
Initial, mg/g DW	420,86±41,10 [b]	490,55±51,45 [b]	30,42±3,26 [a]
Residues after SFE-CO_2	404,14±33,99 [b]	496,67±14,16 [c]	89,15±7,82 [a]
ORAC, TEAC mg TE/g**			
SFE-CO_2 extract:			
mg/g extract	188,43±6,85 [b]	103,41±1,47 [a]	235,11±2,32 [c]
mg/g DW	6,31±0,23 [c]	1,24±0,02 [a]	1,74±0,02 [b]
Plant material:			
Initial, mg/g DW	N/A	N/A	34,89±0,93
Residues after SFE-CO_2	75,18±1,70 [c]	52,25±1,49 [a]	59,33±2,84 [b]

*mg gallic acid equivalents/g. **mg trolox equivalents/g. Different lowercase superscript letters indicate significant differences at $p < 0.05$ (ANOVA, Tukey's test, $p < 0,05$). Values represented as mean ± standard deviation.

3.4. Volatile compounds determination by GCxGC/TOF MS

Gas chromatography time of flight mass spectrometry preliminary analysis of the SFE-CO_2 extract of *G. macrorrhizum* showed 118 peaks. ***Figure 3.4*** shows GC/TOF-MS chromatogram of *G. Macrorrhizum* SFE-CO_2 extract. 21 compounds were characterized and identified by comparison of the mass spectra of the constituents with the NIST library and comparing their calculated retention indexes with those available in literature. All 21 compounds are listed in ***Table 3.6*** with their retention time, name, calculated retention index, retention index from the reference and area. The relative percentage of the chemical compounds of SFE-CO_2 extracts from leaves of G. *macrorrhizum* was expressed as percentage by peak area. For more accurate analysis, injection of authentic standards needs to be done.

Of all constituents detected by GCxGC/TOF MS using comparison of the mass spectra, the most prevailing were waxes (Octacosane, Heptacosane, Hexacosane, etc). From all compounds, waxes consisted more than half of the extract. Due to the CO_2 abilitiy to dissolve other compounds, not only those with high potential activity, waxes are isolated during supercritical carbon dioxide extraction. To avoid waxes in SFE-CO_2 extract, fractional separation could be applied. By operating two or more separators, this method could separate compounds that are in supercritical solution. Due to no mass transfer resistance when waxes and wax-free SFE-CO_2 extracts are in the supercritical solution, solubility data can be applied for separation wax from the other constituents in SFE-CO_2 extract. This process was first reported by Stahl *et al*. [120]. For example, at temperatures around 0 °C terpenes are fully soluble and waxes solubility is near zero. Moreover, for chromatographically separation of extracted compounds, supercritical fluid chromatography (SFC) can be used [121].

One of the waxy solids indicated by MS and RI methods was 9-Octadecenamide (0.10 %, ***Table 3.6***). This compound is classified as food additive and Hypnotics and Sedatives by MeSH (Medical Subject Headings) [122]. Moreover, some sesquiterpenes were detected in SFE-CO_2 extract including trans-β-elemenone (2.58 %), α-curcumene (0.35 %), germacrone (0.13 %), γ-elemene (0.12 %).

Sesquiterpenes. Elemenone has significant antibacterial and antifungal activities [123]. Another sesquiterpene, alpha-curcumene, has anti-inflammatory activity that was reported by Lenfeld *et al.* [25]. Elemene can be used in chemoteraphy due to its anti-proliferative effects toward cancer cells [124]. Germacrone is known for its antiviral and anticancer activity [125]. This sesquiterpene is widely discussed in Literature review of this work.

Chalchat *et al* [126] analysed essential oil of *G. macrorrhizum* obtained via hydro distillation by GC/MS and detected 13 constituents. The dominant compound was sesquiterpene germacrone (37.4 %), lower concentration had (E)-β-elemenone (3.3 %), piperitone (3.0 %) and 2-phenylethyl isovalerate (1.6 %), linalool (0.8 %), β-selinene (0.5 %), geranylacetone (0.5 %), cis-β-elemenone (0.4 %), α-curcumene (0.3 %), β-elemene (0.2 %), α-terpineol + borneol (0.2 %). Among these compounds, Takayasu *et al.* also identified dimethyl sulfide, α-pinene, myrcene and limonene in the geranium species [127]. In comparison with SFE-CO_2 extract, SFE-CO_2 extract lack of monoterpenes such as myrcene, terpineol.

As previously reported by Machado *et al*., monoterpene myrcene has anti-*Leishmania* activity [128]. Chao *et al.* reported that terpineol significantly reduced tyrosinase activity and melanin content. Moreover, this compound decreased levels of malondialdehyde (MDA) [129]. It means that terpineol can be used for melanoma cells.

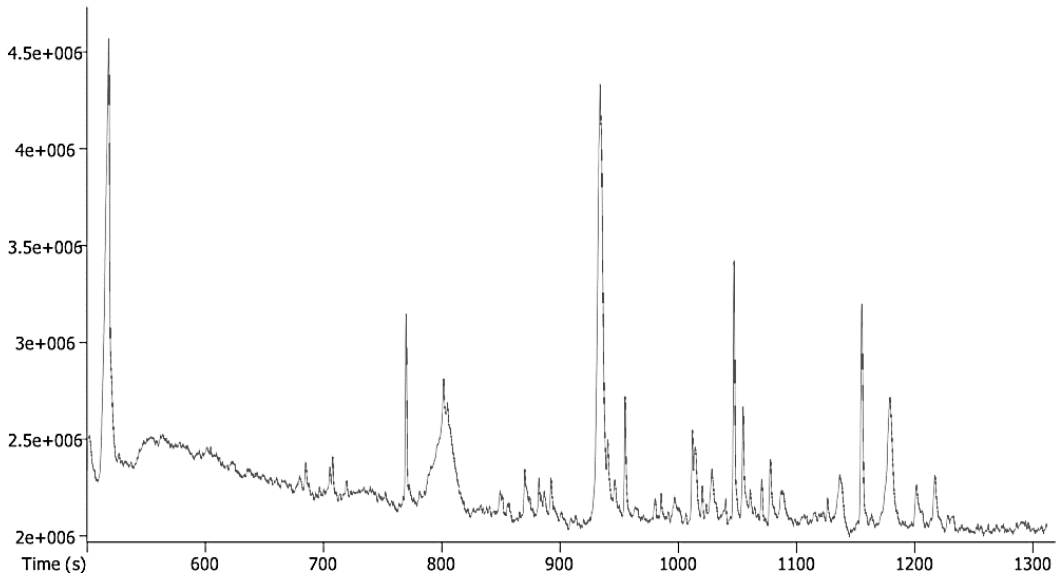

Figure 3.4. GCxGC/TOF MS chromatogram of *G. macrorrhizum* leaves SFE-CO_2 extract

Table 3.6. Results of the GC-MS analysis of SFE-CO_2 extract under optimal conditions

R.T. (s)	Name	Retention Index	Retention Index*	Reference	Area, %
501,1	1-Butanol	1112	1111	Weckerle, B., et al.	2.64
515,8	Maltol	1138	1108	Adams RP., et al.	1.43
684,9	γ–Elemene	1455	1433	Karioti A., et al.	0.12
705,4	α–curcumene	1497	1483	Adams RP, et al.	0.35
752,1	Actinidiolide	1598	1550	Robinson, A., et al.	0.19
767,3	Germacrone	1633	1644	e Morais, S.M., et al.	0.13
769,8	trans-β-Elemenone	1639	1600	Adams RP, et al.	2.58
855,3	2-Tetradecanone	1846	1855	Kubota, K., et al.	0.29
872,6	n-Heptadecanol-1	1890	1941	Muselli, A., et al	0.24
886,5	Geranylacetone	1927	1883	Awano, K., et al.	0.28
886,5	Farnesyl Acetone	1927	1922	Leffingwell JC, et al.	0.28
913,2	Eicosane	2000	2000	von Kovats, E., et al.	0.31
954,9	Phytol	2119	2119	Todua, N.G., et al.	3.95
980,5	Palmitic amide	2195	2150	Kawasaki W., et al.	0.61
1032,3	9-Octadecenamide	2402	2397	von Kovats, E., et al.	0.10
1047,2	Tetracosane	2356	2400	von Kovats, E., et al.	10.02
1070,6	2-Methyltetracosane	2471	2463	von Kovats, E., et al.	0.85
1126,3	Hexacosane	2622	2600	von Kovats, E., et al.	0.85
1155,2	Heptacosane	2693	2700	von Kovats, E., et al.	3.23
1163,4	n-Tetracosanol-1	2711	2710	Zheng, Y., et al.	0.17
1179,1	Octacosane	2745	2800	von Kovats, E., et al.	5.42

*Retention index according to available literature

Concentrations of the germacrone on *G. macrorrhizum* leaves SFE-CO_2 extract are reported in ***Table 3.7.*** Values are expressed as μg/mg of G. MACRORRHIZUM leaves and represent the mean values of two replicates with standard deviation. The highest germacrone content was detected in extract under 350 bar, 50 °C and 15 min (32,2 μg/mg). The lowest germacrone content was detected in extract under 350 bar, 50 °C and 45 min (4,8 μg/mg). Since these both extracts were obtained under same pressure and temperature, the time could be an influence factor here. Some studies say that germacrone is sensitive to temperature [130], thus it could be the factor of germacrone degradation. Curiously, extract obtained under same pressure (350 bars), same time (45 min), but higher temperature (60 °C) had a high content of germacrone (29,5 μg/mg). Due to the fact, that this sesqueterpene could be sensitive not only to the temperature, but to the light and oxygen as well [131], [130] and undergo rearrangements to other sesquiterpenes, storage conditions after extraction could be considered. Moreover, some studies reported that error of results could occur due to gas chromatography analysis conditions [132]. Since, germacrone is heat-sensitive component, it may degradate during GC analysis. Thus, method should be based on stability of analyte. Studies are saying that rearrangement of germacrone to β-elemenone is very common in the GC analysis that is operated under 250 °C [133]. Anyway, since in this GC analysis

the temperature of injection was low (50 °C), the GC analysis is not the case for such a differences in content of germacrone.

Table 3.7. Germacrone content analysis in extract under various conditions

G. macrorrhizum leaves SFE-CO_2 extract	
Conditions (pressure, bar/temperature, °C/time, min)	Germacrone content, μg/mg
350/50/45	4,8±0,7
500/40/75	8,0±0,0
500/50/45	6,9±0,3
350/50/75	5,6±0,6
350/50/15	32,1±3,4
500/60/75	11,4±0,2
200/40/15	7,5±0,4
200/50/45	8,9±0,4
200/60/15	29,5±3,1
200/60/75	22,9±0,3
200/40/75	8,7±1,0
350/60/45	28,4±0,2
500/60/15	9,6±2,1
350/40/45	6,0±0,5

Values presented as mean ± standard deviation.

3.5. Non-volatile compounds determination by UPLC/ESI-Q-TOF-MS

The identification of chemical compounds of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts was based on characteristics of m/z detected under ESI negative ionization mode. ***Tables 3.8, 9, 10*** show the profile of *Geranium machrorrhizu*m leaves, roots and *R. carthamoides* roots SFE-CO_2 extracts, respectively.

SFE-CO_2 extract of *G. macrorrhizum* leaves (*Table 3.8*). In this study, 6 peaks were detected. Comparing their structures, they were similar to wedelolactone, 1,1,3,3-Tetraphenylacetone, 1-(3,4-Dimethoxyphenyl)-5-(5,5-dimethyl-1,3-dioxan-2-yl)-1-pentanone, 3,9-Di(3-cyclohexenyl)-2,4,8,10-tetraoxaspiro[5.5]undecane, (8)-Gingerol and Leukotriene B4. Two compounds (1-(3,4-Dimethoxyphenyl)-5-(5,5-dimethyl-1,3-dioxan-2-yl)-1-pentanone and Leukotriene B4) had the MS score of 100. This score shows the contribution to mass accuracy, isotope abundance and isotope spacing [134].

First detected compound was ascribed to wedelolactone (WDL). It is a natural plant coumestan exhibiting cytotoxicity towards cancer cells. This coumestan inhibits chymotrypsin-like, trypsin-like, and caspase-like proteasome activity in cells of breast cancer [135]. Moreover,

Sarveswaran *et al*. observed that WDL can downregulate the expression of c-Myc mRNA in cells of prostate cancer. Moreover, this anti-inflammatory botanical compound decreases the viability of androgen-sensitive cells of prostate cancer by synergizing with enzalutamide via induction of apoptosis [136]. Ali *et al*. analysed murine skin and reported that this compound can mitigate ultra-violet B radiation induced oxidative stress, which is one of the major dermal pathology factors, as well as events of early tumor promotion [137]. Another important compound that could be assigned to the SFE-CO_2 extract of *G. macrorrhizum* leaves, was (8)-Gingerol. This compound belongs to homologous phenolic ketones. This phenolic ketone exhibits a lot of biological activities, such as antioxidant, anticancer, anti-inflammatory, antimicrobial as well as antiallergic effect [138], [139], [140]. Extract also contained leukotriene B4 (LTB4). LTB4 is pro-inflammatory mediator. Furthermore, leukotriene B4 stimulates the production of a number other pro-inflammatory cytokines mediators that are able to prolong inflammation of tissue [141]. This compound is associated with urtication (hives) after contact with stinging plants [142]. Bioactivity and physiology of leukotrienes have been widely reviewed by Denzlinger [143].

Table 3.8. SFE-CO_2 *Geranium machrorrhizum* leaves

Time	Meas. m/z	Avarage mass, DA*	Suggested formula	Score	Name*
4.9-5.0	313.0372	314.246	$C_{16}H_{10}O_7$	37.45	Wedelolactone
5.7-5.9	361.1598	362.463	$C_{27}H_{22}O$	5.85	1,1,3,3-Tetraphenylacetone
6.2-6.3	335.1864	336.423	$C_{19}H_{28}O_5$	100	1-(3,4-Dimethoxyphenyl)-5-(5,5-dimethyl-1,3-dioxan-2-yl)-1-pentanone
7.0-7.0	319.1924	320.423	$C_{19}H_{28}O_4$	77.71	3,9-Di(3-cyclohexenyl)-2,4,8,10-tetraoxaspiro[5.5]undecane
7.4-7.7	321.2083	322.439	$C_{19}H_{30}O_4$	54.31	(8)-Gingerol
7.8-8.1	335.2221	336.466	$C_{20}H_{32}O_4$	100	Leukotriene B4

*Name and average mass of compounds was suggested by ChemSpider chemical structure database.

SFE-CO_2 extract of *G. macrorrhizum* roots (*Table 3.9*). In this study, 4 compounds were tentatively ascribed. Comparing their structures, they were similar to 4-(Methoxycarbonyl)benzyl4-(benzyloxy)benzoate, Methyl3-{2-[4-(benzyloxy)phenoxy] ethoxy} benzoate, Benzyl β-D-glucopyranoside and Artemotil. The highest MS score had Benzyl β-D-glucopyranoside (71.61). It shows contribution to mass accuracy, isotope abundance and isotope spacing. Luyen *et al*. isolated Benzyl β-D-glucopyranoside in flowers of *Chrysanthemum indicum* [144]. Moreover, this author reported that this compound showed α-glucosidase inhibitory and antioxidant activity [145]. Benzyl alcohol beta-glucopyranoside was also isolated by Wang *et al*.

in flower buds of *Lonicera japonica* [146]. Artemotil belongs to compounds known as amebicides. All amebicides are agents with destructive activity on amebae that causes amebiasis in animal and man [122]. Artemisinin derivative artemotil is widely used against malaria [147]. This compound is able to do a combination between rapid blood schizonticide activity and wide therapeutic index. Artemitol appears to be quite safe [148].

Table 3.9. SFE-CO_2 *Geranium machrorrhizum* roots

Time	Meas. m/z	Avarage mass, DA*	Suggested formula	Score	Name*
2.3-2.4	375.1250	376.131	C23H20O5	2.14	4-(Methoxycarbonyl)benzyl 4-(benzyloxy)benzoate
2.8-2.9	377.1401	378.418	C23H22O5	2.25	Methyl 3-{2-[4-(benzyloxy)phenoxy]ethoxy}benzoate
5.8-5.8	269.1020	270.278	C13H18O6	71.61	Benzyl β-D-glucopyranoside
6.1-6.2	311.1886	312.401	C17H28O5	34.41	Artemotil

*Name and average mass of compounds was suggested by ChemSpider chemical structure database.

SFE-CO_2 extract of *R. carthamoides* roots (*Table 3.10*). In this study 1 compound were analyzed at the MS score of 67.83. Comparing its structure, compounds were similar to artemotil. As it was mention before, this chemical compound has a strong potential activity against malaria.

In ethanol or aqueous extracts of *R. carthamoides* Miliauskas *et al*, reported various flavonoids. Author isolated Quercetagetin-7-O-β- glucopyranoside, 6-Hydroxykaempferol-7-O-β-glucopyranoside, quercetagetin-7-O -(6″-Oacety l-β- glucopyranos ide 6-Methoxykaempferol-3-O-β-glucopyranoside and 6- Hydroxykaempferol-7-O-(6’’-O-acetyl-β-D-glucopyranoside) [149].

Table 3.10. SFE-CO_2 *R. carthamoides* roots

Time	Meas. m/z	Avarage mass, DA*	Suggested formula	Score	Name*
5.5-5.5	311.1878	312.401	C17H28O5	67.83	Artemotil

*Name and average mass of compounds was suggested by ChemSpider chemical structure database.

3.6. Conventional extraction techniques. Solid-liquid extraction (SLE)

The effect of the solvent type on the extraction yield under the same extraction conditions (time, temperature, particle size) was examined (***Table 3.11***). The results showed that different solvents significantly affected the extraction yield. It is the evident that extraction yield of *Geranium machrorrhizum* leaves, roots and *Rhaponticum carhtamodes* roots was the highest using

ethanol as a solvent. The highest extraction yield was obtained on *G. machrorrhizum* roots (16.69 %) using ethanol and lower on *R. carthamoides* roots (3.68 %). The lowest extraction yield was obtained on *R. carthamoides* roots (0.37 %) using hexane. The extraction yield with hexane on *G. macrorrhizum* roots was slightly higher (1 %). However, when hexane was changed to acetone, the extraction yield was increased in *G. macrorrhizum* and *R. carthamoides* roots (9.58 and 1.39 %, respectively).

In comparison between Soxhlet extraction with hexane and solid-liquid extraction, Soxhlet extraction yield was higher. SLE-He extraction yield was the lowest. For example, *G. Macrorrhizum* roots Sox-He and SLE-He extraction yield was two times higher (1.00 and 1.91 %, respectively). In comparison between *G. macrorrhizum* leaves and roots non-polar fraction extraction yields, the highest lypophilic extract was obtain using Soxhlet extracion method using hexane as a solvent. The lowest – using solid-liquid extraction technique in a shaker (1.4 and 1.9 times lower, respectively). Here the impact of temperature could be concidered. In Soxhlet-He temperature was 80 °C, in SLE – 20 °C (room temperature). Majeed *et al.* reported the extraction yield from *O. vulgare* leaves using water and methanol in different ratios as a solvent [149]. Extraction yield ranged from 5.32 to 14.65 %. *O. Vulgare* leaves extraction yield under solvent ratio 70:30 (methanol:water) (5.55 %) was similar to *G. macrorrhizum* leaves extraction yield using 100 % of ethanol (5.53 %).

Moreover, as shown in ***Table 3.11***, the yield of extract of *G. macrorrhizum* leaves and roots obtained by Soxhlet extraction technique using hexane was higher than those obtained by SFE-CO_2. SFE-CO_2 extraction yields for leaves and roots under optimised conditions reached 84 and 63 % Sox-He values. Moreover, SFE-CO_2 extraction yields were higher 15 and 20 % than SLE-He extraction yields, respectively. Furthermore, using high pressure extraction technique for *G. Macrorrhizum* leaves the time was 7 times longer than in conventional extraction techniques (52 vs 360 min, respectively). The highest lypophylic fraction from *R.carthamoides* roots was obtaines using SFE-CO_2 extraction technique: ~1.2 and 2 times higher than Sox-He and SLE-He, respectively.

Overall, 8.89 % of *G.macrorrhizum* leaves, 27,27 % of *G.macrorrhizum* roots and 5,44 % of *R.carthamoides* roots soluble parts were obtained using step-by-step SLE extraction method with different polarity solvents. The highest soluble parts were obtained using acetone as a solvent. It was 26-35% and 61-68% of total extraction yield. Hexane (non-polar) fraction were obtained in this order: *G.macrorrhizum* leaves - 33% > *R.carthamoides* roots - 7% > *G.macrorrhizum* roots – 4%.

Table 3.11. *G. macrorrhizum* leaves, roots and *R. carthamoides* roots polar and non-polar extraction yields using different extraction methods

Extraction yield, %	Extraction parameters			*G. macrorrhizum*		*R. carthamoides*
	P, bar	T, °C	τ, min	***Leaves***	***Roots***	***Roots***
Supercritical carbon dioxide extraction (SFE-CO_2):						
SFE-CO_2	462	55	52	3,41*	-	-
SFE-CO_2	462	55	720	-	1,20*	-
SFE-CO_2	462	55	420	-	-	0,74*
Soxhlet extraction (Sox):						
Sox-He	1	80	360	$3,97 \pm 0,03^{c}$	$1,91 \pm 0,02^{b}$	$0,67 \pm 0,04^{a}$
Solid-liquid extraction (SLE):						
SLE-He	1	20	360	$2,92 \pm 0,23^{c}$	$1,00 \pm 0,03^{b}$	$0,37 \pm 0,03^{a}$
SLE-Act	1	20	360	$2,62 \pm 0,19^{b}$	$9,58 \pm 0,13^{c}$	$1,39 \pm 0,13^{a}$
SLE-EtOH	1	20	360	$5,53 \pm 0,14^{b}$	$16,69 \pm 1,30^{c}$	$3,68 \pm 0,21^{a}$

*Large pilot scale supercritical carbon dioxide extractor. Different lowercase superscript letters indicate significant differences at $p < 0.05$ (ANOVA, Tukey's test, $p < 0,05$). Values represented as mean ± standard deviation.

3.7. Downstream valorization (bio-refinery process)

Nowadays large amount of food industry-related waste raises environmental and economic problems, thus reusability of by-products is a hot topic [150]. Bio-refinery process can combine technologies between raw materials and final products and refine corresponding materials to valuable products [151]. Here, the combination of supercritical carbon dioxide extraction followed by pressurized liquid extraction formulate a downstream extraction scheme. Pressurized liquid extraction was employed to recover polar bioactive compounds from *G. macrorrhizum* leaves that have been already defatted by supercritical carbon dioxide extraction (***Figure 3.5***).

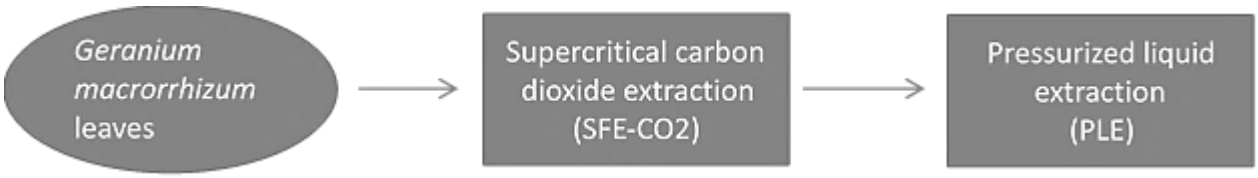

Figure 3.5. Downstream valorization of *G. macrorrhizum* leaves

3.7.1. Experimental design of pressurized liquid extraction

CCD experimental design using response surface methodology (RSM) was proposed to study the effect of two parameters, percentage of ethanol in the solvent mixture (0-100%) (v/v) and temperature (50-170 ºC), on a pressurized liquid extraction. By using a 3-level factorial design 3^2 the effect on extraction yield, total phenolic content (mg GAE/g extract), $DPPH^{\bullet}$ radical scavenging (EC_{50}, μg/ml) and antioxidant capacity (mM Trolox/g extract) was studied in order to maximize it (***Figure 3.6)***. Based on previous studies the rest of extraction conditions remained

constant [56] [98]. Static extraction was 20 min, pressure was 10.3 MPa. In total, 20 experiments were carried out in randomized order. Data were analyzed via ANOVA. It was detected that modifications of the extraction conditions had the effect on the response variables. The whole experiment design and results of experiment are presented in ***Table 3.16.***

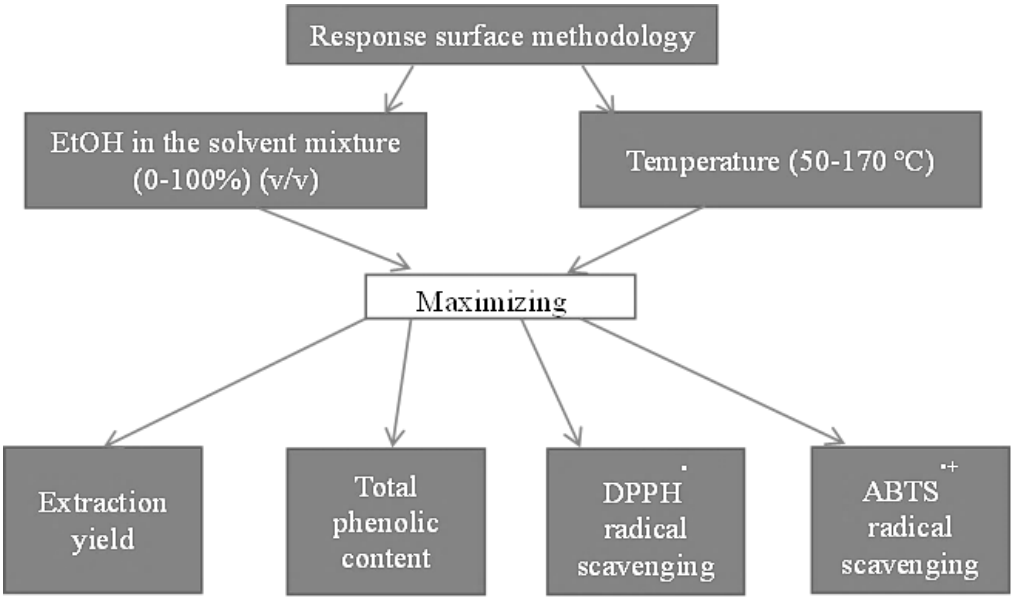

Figure 3.6. Experimental design of *G. macrorrhizum* fat-free leaves

3.7.2. Effect of temperature and solvent on extraction yield

The ANOVA table (***Table 3.12***) partitions the variability of the extraction yield into separate pieces for each of the factors. It tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, 4 effects have P-values less than 0.05 (temperature, percentage of EtOH in the solvent mixture, quadratic variable of temperature and quadratic variable of EtOH) indicating that they are significantly different from zero at the 95.0% confidence level. The PLE extraction yield was not significantly affected by the temperature and interaction between temperature and percentage of ethanol in the solvent mixture, as can be seen in ***Table 3.12.*** The R-Squared statistic indicates that the model as fitted explains 98.9464% of the variability in extraction yield. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 98.4602%. The standard error of the estimate shows the standard deviation of the residuals to be 1.94224. The mean absolute error (MAE) of 1.25794 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in the data file. Since P-value is greater than 5.0%, there is no indication

of serial autocorrelation in the residuals at the 95.0% significance level. The regression equation which has been fitted to the model is presented below (***Equation 15***):

$$Yield, \% = 25.4381 + 0.0893643 * T + 0.351939 * E + 0.000461542 * T^2 - 0.00606655 * E^2 - 0.000009295 * T * E \text{ , (15)}$$

where: T is temperature, E is percentage of ethanol in the solvent mixture

Table 3.12. Analysis of Variance for extracton yield

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	1566.73	1	1566.73	415.54	0.0000*
B:%EtOH	1962.07	1	1962.07	520.39	0.0000*
AA	12.8835	1	12.8835	3.42	0.0874*
AB	0.00622059	1	0.00622059	0.00	0.9682
BB	1073.42	1	1073.42	284.70	0.0000*
blocks	0.140109	1	0.140109	0.04	0.8501
Total error	49.0148	13	3.77037		
Total (corr.)	4654.98	19			

*Significant value (p<0.05). R-squared = 98.947 percent; R-squared (adjusted for d.f.) = 98.4611 percent; Standard Error of Est. = 1.94174; Mean absolute error = 1.25747; Durbin-Watson statistic = 1.86059 (P=0.2887); Lag 1 residual autocorrelation = 0.0676943

The extraction yield was increasing at higher temperatures using all solvent mixtures. The significance of temperature on extraction yield is supported by standardised Pareto chart (***Figure 3.7)***. Vertical line on the plot shows the effects that are significant at 99 % confidence level [152] [153]. Results illustrated in Pareto chart also show that EtOH percentage in solvent mixture (B) has negative effect on the yield. Corresponding response surface for the temperature versus the ratio of EtOH in the solvent mixture is shown in ***Figure 3.8***. Analysis of the generated response surfaces revealed that the highest yields were obtained using higher temperatures.

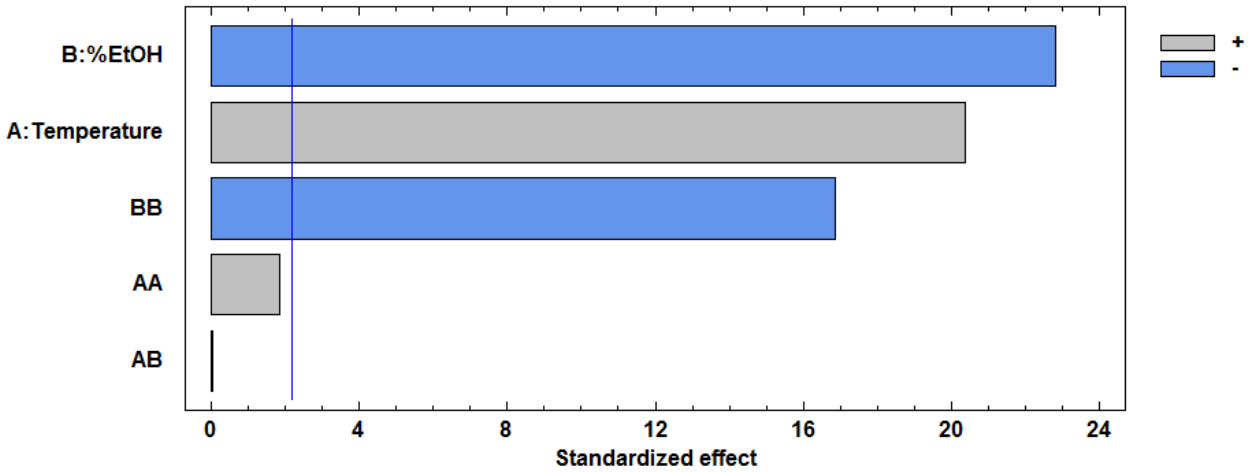

Figure 3.7. Standartized Pareto chart for the yield response variables studied in the experimental design. Grey bar shows positive effect, blue bar shows negative effect**.**

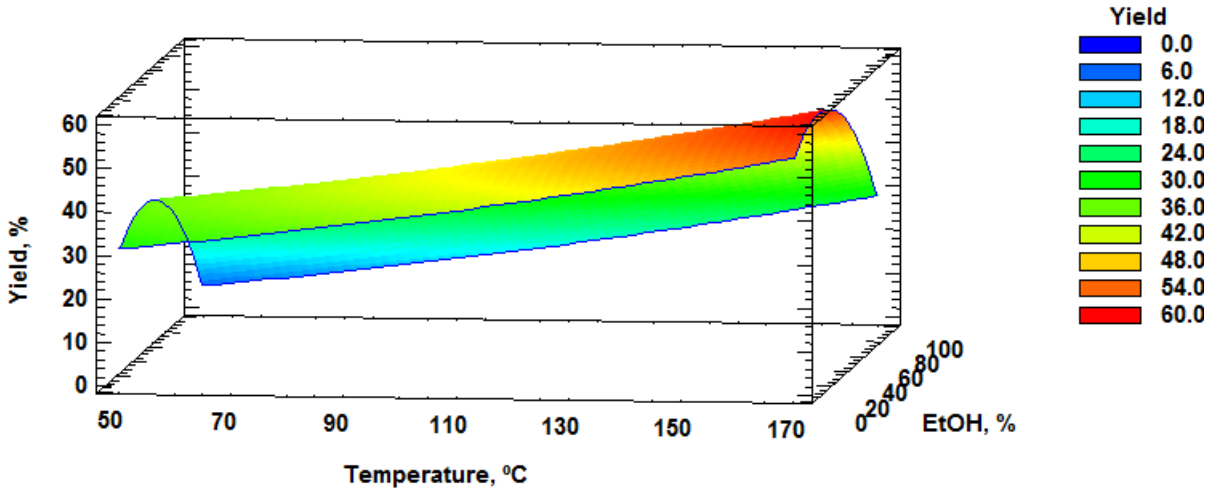

Figure 3.8. Corresponding response surfaces of extraction yield in the experimental design

The values of extraction yield ranged between 5.52 and 59.89 % under different conditions. Overall, the maximum yield was obtained using 50 % of ethanol at 170 ºC (59.89; 57.22 %). Brazauskas *et al*. observed [98] *A. melanocarpa* fat-free pomace PLE extracts. Authors reported that under 170 ºC and 50 % of ethanol with 2% of formic acid obtained the highest extraction yield (75.66 %). At the same temperature and percentage of ethanol PLE performed on *G. macrorrhizum* fat-free leaves extract showed lower extraction yield (59.89; 57.22 %). Petlevski *et al.* perfomed an extraction of *Pelargonium radula* (*Geraniaceae* family) by heating (100 ºC) dry leaves for 2 h in water [154]. Obtained extraction yield (33.2 %) was lower than pressurized extraction yield under 110 ºC using water as a solvent (41.38-43.20 %).

3.7.3. Effect of extraction conditions on total phenolic content (Folin-Ciocalteu method)

Total phenolic content of the PLE extracts is expressed as milligrams of gallic acid equivalents per gram. According to ANOVA (***Table 3.13***) one effect, the quadratic variable of temperature, had a P-value less than 0.05 thus it is significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 58.9874% of the variability in TPC. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 40.0586%. It is not a high R-squared value. Since the coefficient is significant, one unit shift in the independent quadratic variable of temperature gives the mean change in the dependent variable of total phenolic content. The standard error of the estimate shows the standard deviation of the residuals to be 38.4021. The mean absolute error (MAE) of 25.0586 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the P-value is greater than 5.0%, there is no

indication of serial autocorrelation in the residuals at the 5.0% significance level. The regression equation which has been fitted to the model is presented below (***Equation 16***):

$$TPC, mg\frac{GAE}{g} = 197.294 + 3.27361 * T + 0.410318 * E - 0.0167434 * T^2 - 0.0107338E^2 + 0.00548264 * T * E \text{ , (16)}$$

where: T is temperature, E is percentage of ethanol in the solvent mixture

Table 3.13. Analysis of Variance for total phenolic content

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	796.867	1	796.867	0.54	0.4753
B:%EtOH	107.883	1	107.883	0.07	0.7910
AA	16955.1	1	16955.1	11.50	0.0048*
AB	2164.27	1	2164.27	1.47	0.2473
BB	3360.4	1	3360.4	2.28	0.1551
blocks	1020.74	1	1020.74	0.69	0.4205
Total error	19171.3	13	1474.72		
Total (corr.)	46745.1	19			

*Significant value ($p<0.05$). R-squared = 58.9874 percent; R-squared (adjusted for d.f.) = 40.0586 percent; Standard Error of Est. = 38.4021; Mean absolute error = 25.0586; Durbin-Watson statistic = 2.75376 (P=0.9299); Lag 1 residual autocorrelation = -0.445094.

The Pareto chart (***Figure 3.9)*** showed a strong negative effect of the quadratic variable of temperature which was significant at 99% confidence level. In Pareto chart vertical line on the plot shows the effects that are significant [152] [153]. Corresponding response surface for the temperature versus the ratio of EtOH in the solvent mixture is shown in ***Figure 3.10***. Analysis of the generated response surfaces revealed that the highest responses were obtained using temperatures and percentage of ethanol in the solvent mixture of the middle point (~50 ºC and 50 %, ETOH).

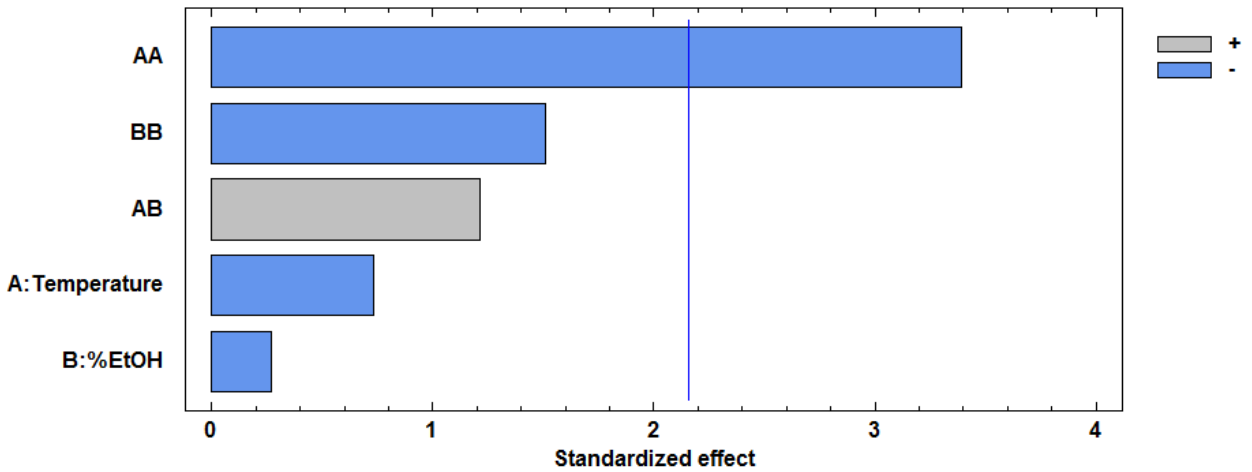

Figure 3.9. Standartized Pareto chart for the total phenolic content response variables studied in the experimental design. Grey bar shows positive effect, blue bar shows negative effect**.**

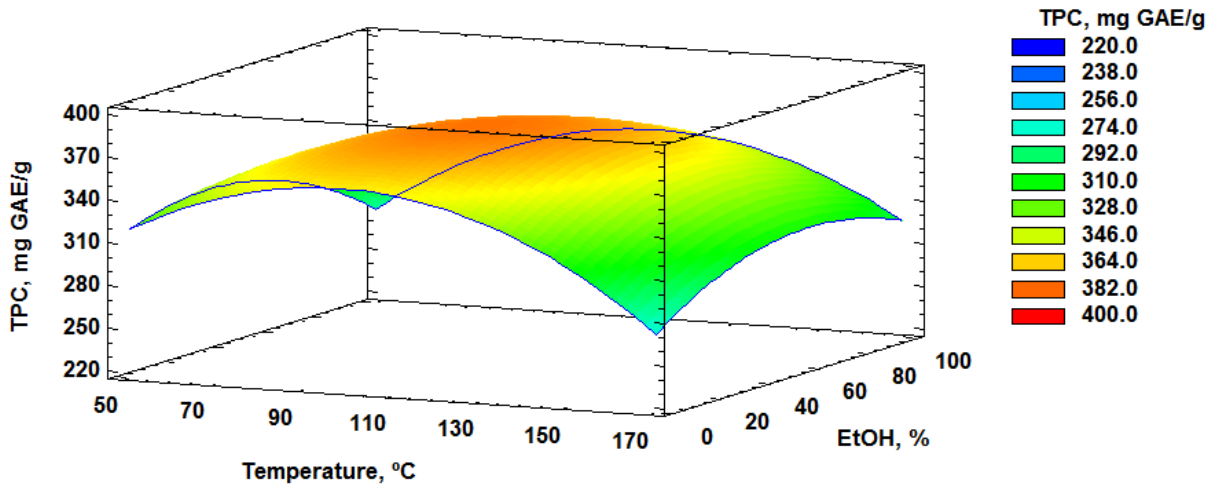

Figure 3.10. Corresponding response surfaces of total phenolic content in the experimental design

The values of TPC ranged between 226.44 and 399.09 mg GAE/g of all pressurized liquid extracts under different extraction conditions. The extracts obtained by pressurized liquid extraction with 50% ethanol under 50 ºC (399.09 mg GAE/g) and 110 ºC (389.37 mg GAE/g) respectively showed the highest total phenolic content. It appears that the solvent mixture of 50 % ethanol was the best for the extraction of phenolic compounds. Alhough, Brazauskas *et al*. [98] reported higher PLE yield (75.66 %) of black chokeberries than PLE of *G. macrorrhizum* (59.89 %), total phenolic content was higher in G. MACRORRHIZUM extract (227.24, 328.2 mg GAE/g, respectively). It means that the yield of black chokeberries was shifted by not phenolic but other compounds. Deepika *et al.* reported that the 100 % methanolic extract of *P. zonale* (*Gerancieae* family) obtained in a shaker extractor under 37 ºC TPC content was 24 mg GAE/g [155]. Pressurized liquid extract total phenolic content of *G. macrorhizum* under 50 ºC was much higher (226.44-278.15 mg GAE/g).

3.7.4. Effect of extraction conditions on DPPH• radical scavenging activity

The radical scavenging capacity of extracts was measured by the DPPH• free radical assay. This assay is known as a sensitive way to determine radical scavenging [113]. The ANOVA table (***Table 3.14***) shows that 3 effects have P-values less than 0.05 (quadratic variables of temperature and EtOH, interaction between temperature and EtOH), indicating that they are significantly different from zero at the 95.0% confidence level. The R-Squared statistic indicates that the model as fitted explains 72.5517% of the variability in EC50. The adjusted R-squared statistic is 59.8833%. The standard error of the estimate shows the standard deviation of the residuals to be 0.487845. The mean absolute error (MAE) of 0.324327 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation

based on the order in which they occur in your data file. Since the P-value is greater than 5.0%, there is no indication of serial autocorrelation in the residuals at the 5.0% significance level. The regression equation which has been fitted to the model is presented below (***Equation 17***):

$$EC_{50}, \frac{\text{ug}}{\text{mL}} = 5.22753 - 0.0275377 * T - 0.00518975 * E + 0.000141679 * T^2 - 0.000284174 * E^2 - 0.000162995 * T * E \text{ , (17)}$$

where: T is temperature, E is percentage of ethanol in the solvent mixture

Table 3.14. Analysis of Variance for EC_{50}

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	0.881812	1	0.881812	3.71	0.0764
B:%EtOH	0.842138	1	0.842138	3.54	0.0825
AA	1.21401	1	1.21401	5.10	0.0417*
AB	1.91284	1	1.91284	8.04	0.0141*
BB	2.35535	1	2.35535	9.90	0.0077*
blocks	0.289945	1	0.289945	1.22	0.2897
Total error	3.0939	13	0.237993		
Total (corr.)	11.2717	19			

*Significant value ($p<0.05$). R-squared = 72.5517 percent; R-squared (adjusted for d.f.) = 59.8833 percent; Standard Error of Est. = 0.487845; Mean absolute error = 0.324327; Durbin-Watson statistic = 2.92494 (P=0.9720); Lag 1 residual autocorrelation = -0.533538

Figure 3.11 illustrates the standardized Pareto chart for experimental design. Vertical line on the plot shows the effects that are significant at 99 % confidence level [152] [153]. According to ***Figure 3.11***, interaction between temperature and percentage of EtOH in the solvent mixture was the most significant factor having a negative effect on the EC_{50} in this study. Thus, a positive effect on antioxidant activity. It was observed that quadratic effect of temperature and EtOH ratio in the solvent mixture is significant and has a positive effect. Corresponding response surface for the temperature versus the ratio of EtOH in the solvent mixture is shown in ***Figure 3.12***. Analysis of the generated response surfaces revealed that the lowest responses were obtained using temperatures and percentage of ethanol in the solvent mixture of the middle point (~50 ºC, 50 %, respectively) and highest response surfaces (low antioxidant activity) at high temperatures and low concentration of ethanol in the solvent mixture.

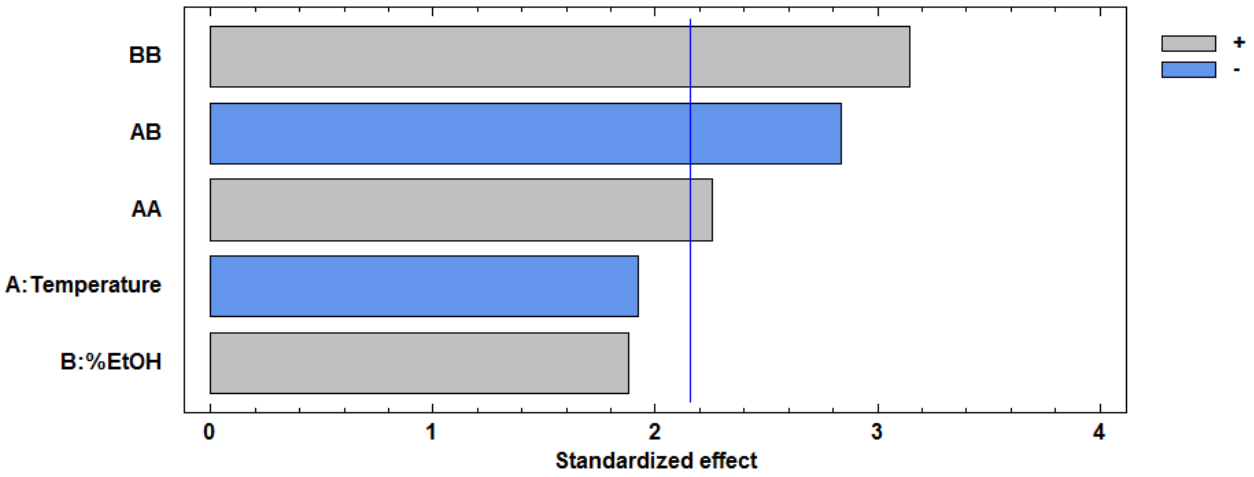

Figure 3.11. Standartized Pareto chart for the EC_{50} response variables studied in the experimental design. Grey bar shows positive effect, blue bar shows negative effect**.**

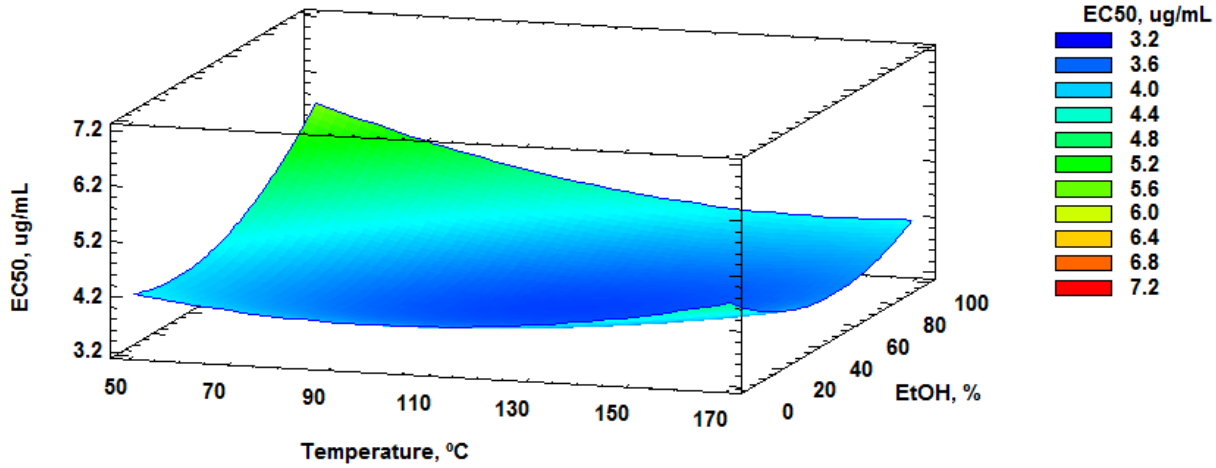

Figure 3.12. Corresponding response surfaces of EC_{50} in the experimental design

Thus, assay results showed that all the extracts have antioxidant properties. The 50% inhibitory concentration (EC_{50}) of *G. macrorrhizum* leaves PLE extracts varied from 3.22 to 6.27 µg/mL depending on extraction conditions. The variations of scavenging activities of the different extracts could be attributed to prevailing antioxidant molecules such as phenols [1]. It was observed that extracts obtained using EtOH as a co-solvent in various concentrations have higher scavenging activity than extracts obtained with water only. Extract obtained under 110 ºC and 50 % of EtOH (middle point) had lowest EC_{50} value (3.22 µg/mL) and the best antioxidant activity. Brazauskas *et al*. observed [98] *A. melanocarpa* fat-free pomace PLE extracts. Authors reported that under 110 ºC and 50 % of ethanol, with 1% of formic acid EC_{50} value (5.06 µg/ml) was the lowest and had highest antioxidant activity. Under similar conditions (110 ºC; 50 % of ethanol) PLE performed on *G. macrorrhizum* fat-free leaves extract showed lower EC_{50} value (3.22-4.04 µg/ml), thus had higher antioxidant activity. Sompaga *et al*. [156] performed Soxhlet extraction on *P. graveolens* (family of Geraniaceae) and maceration extraction techniques using ethyl acetate

and methanol solvents. Authors reported that EC_{50} value (47.666 μg/ml) of the Soxhlet extract using ethyl acetate was the lowest and had highest free radical activity. In our experimental design even the extract with the highest EC_{50} (6.27 μg/ml) had lower EC_{50} value than an extract of *P. graveolens* obtained by Soxhlet. Thus, it could be suggested that PLE extract of Geraniaceae family have higher antioxidant activity than Soxhlet and maceration extracts. In the $DPPH^{\bullet}$ assay, a comparison of EC_{50} of aqueous extracts by Soxhlet [115] of *P. graveolens* (Geraniaceae family) leaves and aqueous extracts by PLE of *G. macrorrhizum* fat-free leaves showed significant difference. Indeed, PLE extracts possessed greater $DPPH^{\bullet}$ radical scavenging activity (3.92-4.56 μg/ml, depending on temperature) than Soxhlet extract (16.59 μg/ml). Ethanolic (70%) extract of the leaves of *R. carthamoides* (EC_{50} = 46 mg/mL) [114] had lower activity than all the PLE extracts of the leaves of *G. macrorrhizum* (3.22 to 6.27 μg/ml, depending on PLE conditions). Petlevski *et al.* perfomed an extraction of *Pelargonium radula* (*Geraniaceae* family) by heating (100 ºC) dry leaves for 2 h in water [154]. Obtained EC_{50} value of the extract (38.9 μg/ml) was higher than pressurized extraction yield under 110 ºC using water as a solvent (3.92 – 4.18 μg/ml). Thus, PLE extract has higher free radical scavenging activity.

3.7.5. Effect of extraction conditions on $ABTS^{\bullet+}$ cation radical scavenging capacity

The radical scavenging capacity of the extracts was also measured by the ability to scavenge $ABTS^{\bullet+}$ radical cation. TEAC of the PLE extracts was expressed as mmol Trolox equivalents per gram. The ANOVA table (***Table 3.15***) shows that 2 effects have P-values less than 0.05, quadratic variable of temperature, interaction between temperature and EtOH, indicating that they are significantly different from zero at the 95.0% confidence level. The $ABTS^{\bullet+}$ radical cation scavenging capacity in PLE extracts was not significantly affected by the temperature and percentage of ethanol in the solvent mixture itself, as can be seen in ***Table 3.15.*** The R-Squared statistic indicates that the model as fitted explains 73.4026% of the variability in TEAC. The adjusted R-squared statistic is 61.1269%. The standard error of the estimate shows the standard deviation of the residuals to be 0.878755. The mean absolute error (MAE) of 0.559821 is the average value of the residuals. The Durbin-Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the P-value is greater than 5.0%, there is no indication of serial autocorrelation in the residuals at the 5.0% significance level.

The regression equation which has been fitted to the model (***Formula 18***):

$$TEAC, \text{mmol TE/g} = 6.0342 + 0.0678219 * T - 0.0307751 * E - 0.000425092 * T\text{^}2 + 0.000429799 * T * E - 0.000153923 * E\text{^}2 \text{ , (18)}$$

where: T is temperature, E is percentage of ethanol in the solvent mixture

Table 3.15. Analysis of Variance for TEAC

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Temperature	0.765131	1	0.765131	0.99	0.3377
B:%EtOH	0.0369974	1	0.0369974	0.05	0.8301
AA	10.9289	1	10.9289	14.15	0.0024*
AB	13.3004	1	13.3004	17.22	0.0011*
BB	0.691024	1	0.691024	0.89	0.3614
blocks	0.707899	1	0.707899	0.92	0.3558
Total error	10.0387	13	0.772211		
Total (corr.)	37.7433	19			

*Significant value ($p<0.05$). R-squared = 73.4026 percent; R-squared (adjusted for d.f.) = 61.1269 percent; Standard Error of Est. = 0.878755; Mean absolute error = 0.559821; Durbin-Watson statistic = 2.78575 (P=0.9400); Lag 1 residual autocorrelation = -0.461529

Figure 3.13 illustrates the standardized Pareto chart for experimental design. Vertical line on the plot shows the effects that are significant at 99 % confidence level [152] [153]. According to ***Figure 3.13***, interaction between temperature and percentage of EtOH in the solvent mixture was the most significant factor having a positive effect on the antioxidant activity in this study. The results illustrated in Fig. 1 also confirm that the quadratic effect of temperature is significant and has the negative effect. Corresponding response surface for the temperature versus the ratio of EtOH in the solvent mixture is shown in ***Figure 3.14*** Analysis of the generated response surfaces revealed that the lowest responses were obtained using high temperatures and low percentage of ethanol in the solvent mixture as well as low temperatures and high percentage of ethanol in the solvent mixture.

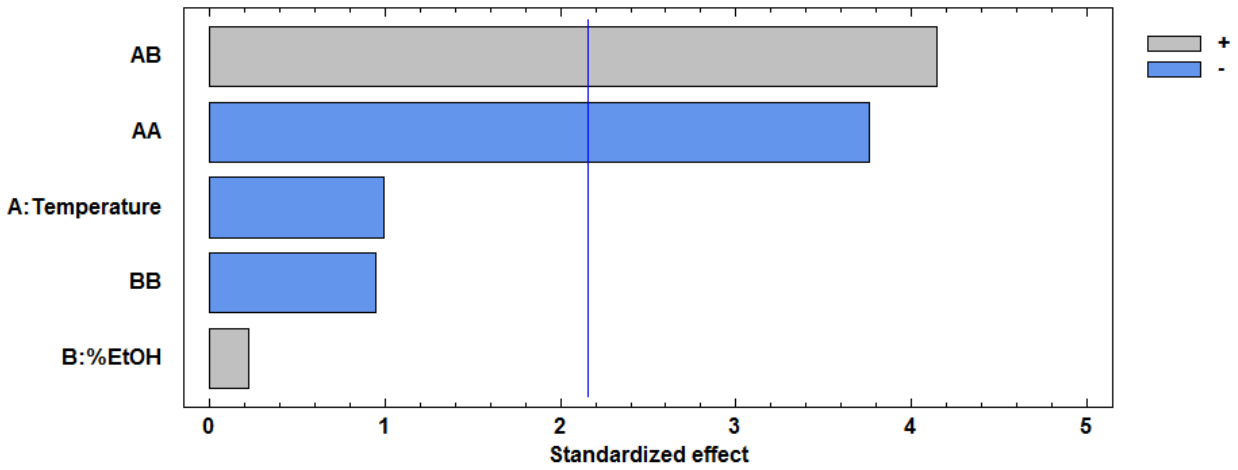

Figure 3.13. Standartized Pareto chart for the TEAC response variables studied in the experimental design. Grey bar shows positive effect, blue bar shows negative effect.

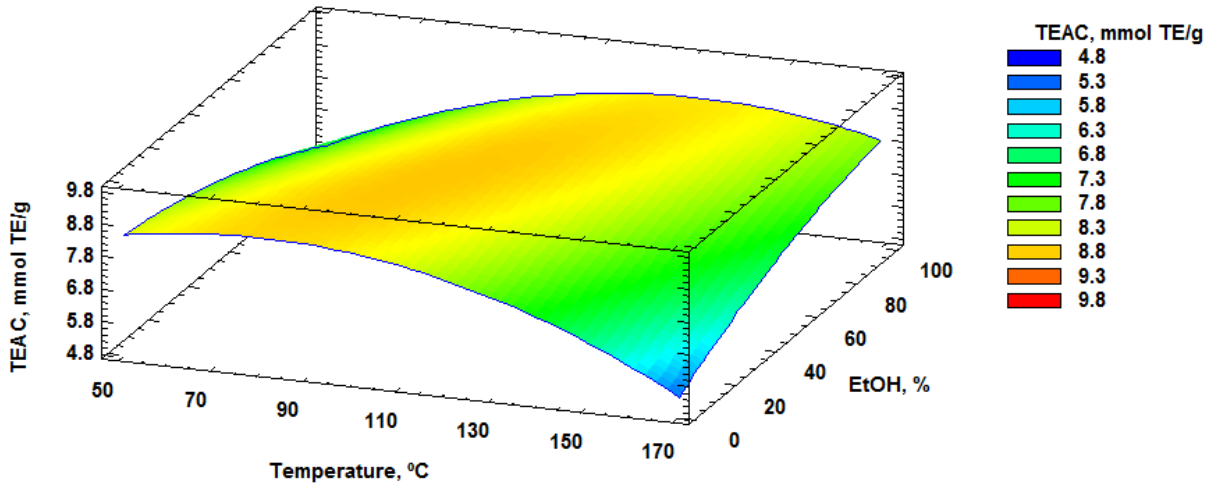

Figure 3.14. Corresponding response surfaces of TEAC in the experimental design

Thus, assay results showed that all the extracts have antioxidant properties. The TEAC value of *G. macrorrhizum* leaves PLE extracts varied from 4.85 to 9.79 mmol TE/g depending on extraction conditions. Extract obtained under 110 ºC and 100 % of EtOH had the highest TEAC value (9.76; 9.79 mmol TE/g) and the best antioxidant activity. Alhough, Brazauskas *et al*. [98] reported higher PLE maximum yield (75.66 %) of black chokeberries than PLE of *G. macrorrhizum* (59.89 %), G. MACRORRHIZUM extract under those conditions had higher antioxidant activity (4.32, 6.85 mmol TE/g, respectively). Results obtained by TPC, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ analysis show that the yield of black chokeberries was shifted by compounds that are not phenols and have lower antioxidant activity.

Table 3.16. Experimental design conditions and response of each extract studied for PLE optimization of *G. macrorrhizum* fat-free leave material

	BLOCK	PLE extraction conditions		Response variables			
		Temperature, ºC	Ethanol, %	Extraction yield, %	TPC, mg GAE/g[b]	EC50, µg/mL[c]	TEAC, mmol TE/g[d]
1	1	110	0	41.38	386.51	4.18	7.87
2[a]	1	110	50	42.38	333.92	3.83	7.09
3	1	170	0	54.51	285.01	4.52	5.37
4	1	170	50	59.89	328.20	3.89	6.85
5	1	50	0	29.79	286.47	4.56	8.44
6[a]	1	110	50	41.04	389.37	3.72	8.53
7	1	170	100	27.74	271.03	4.57	7.35
8	1	50	50	32.63	399.09	3.68	8.42
9	1	50	100	5.98	278.15	6.27	4.85
10	1	110	100	16.79	377.36	3.99	9.76
11	2	110	0	43.20	363.64	3.92	8.28
12[a]	2	110	50	40.98	328.77	4.04	8.71
13	2	170	0	50.84	257.28	4.22	5.82
14	2	170	50	57.22	300.19	3.87	7.49
15	2	50	0	32.04	308.76	4.13	8.21
16[a]	2	110	50	44.18	355.64	3.22	8.76
17	2	170	100	27.06	312.19	4.02	7.62
18	2	50	50	34.18	352.78	3.52	8.35
19	2	50	100	5.52	226.44	6.18	5.27
20	2	110	100	15.24	386.51	3.67	9.79

[a] Central points of experimental design; [b] mg gallic acid equivalents/g extract; [c] DPPH assay efficient concentration (ug/ml); [d] mmol trolox equivalents/g extract

3.7.6. Verification of predictive model of multiple response optimization

Extraction yield increased at higher temperatures using all solvent mixtures. Unfortunately, further analysis of extracts antioxidant activity showed that higher temperature led potentially to degradation of bioactive compounds. According to Pareto chart of extraction yield, higher EtOH percentage in the solvent has a negative effect on extraction yield. Although, Pareto charts of *in vitro* determinations doesn't show significant negative effect of percentage of EtOH. The difference between these results can be affected by water soluble compounds such as polysaccharides that are increasing the extraction yield but don't have a high bioactivity. Further investigations on comparison of LC results between water and ethanol extracts are needed to confirm this theory. Furthermore, some phenols, such as p-coumaric acid or catechin, that are found in *Geraniaceae* family plants [157], [158], are more soluble in ethanol than water [159], [160]. In addition, Rocha *et. al.* reported that ethanolic extracts are richer in flavonoids. Many studies have suggested that flavonoids are able to scavenge free radicals [161], [162]. The presence of flavonoids in *Geranium* are supported by several studies [163], [164]. However, in order to get optimal conditions and obtain an extract with high activity, extraction yield was excluded from multiple response optimization. In the present study the good correlation between TPC and $DPPH^{\bullet}$ as well as $ABTS^{\bullet}$ radical scavenging activity indicated that phenolic compounds are powerful scavengers of free radicals. The strong correlation between these variables is also reported in previous studies [1], [165].

A multiple response optimization was carried out assuming similar statistical weight of all three response variables (TPC, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$) to determine the most suitable conditions of extraction to extract with high TPC and $ABTS^{\bullet+}$ values and low EC_{50} value. To optimize complex experimental processes, RSM is an effective statistical method [166].

The overlay plot for the optimal region is presented in ***Figure 3.15.*** 2D contour plot of estimated response surface (***Figure 3.16.***) is the graphical representations of the regression equation. Middle point is showing optimal conditions of PLE obtained after multiple response optimization. The following optimum extraction conditions were suggested by the model: 107 ºC temperature and 43 % of ethanol in the solvent mixture. To determine the validity of predictive model, predicted and experimental values were compared in ***Table 3.17***. The validity of the obtained model can be approved, because predicted and actual values are in a good agreement. Optimize desirability value is 0.866973. Extracts were produced in high yields and possessed strong bioactivity. Thus, bio-refinery concept can be applied on SFE-CO_2 by-products of *G. macrorrhizum* leaves to get valuable bioactive compounds by pressurized liquid extraction.

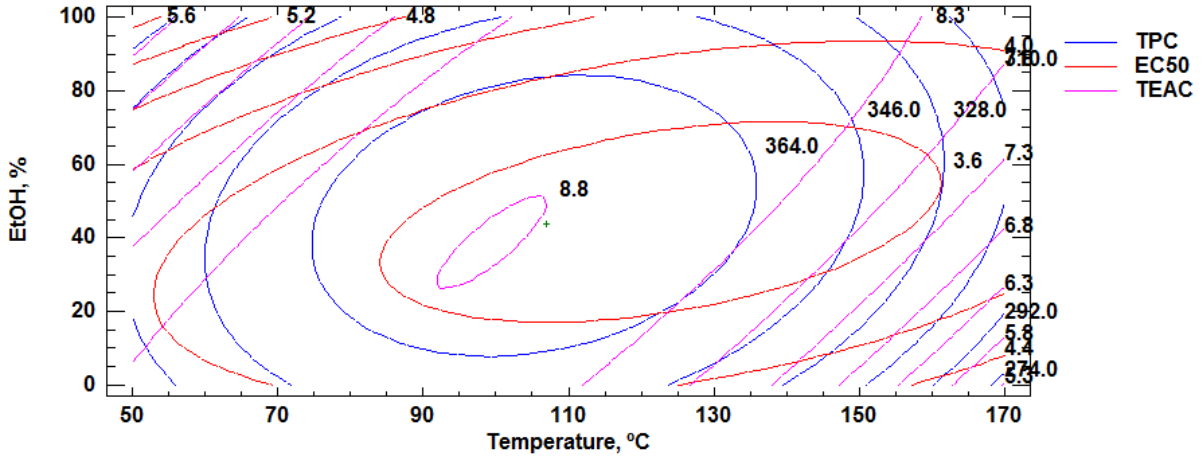

Figure 3.15. Overlay plot of multiple region

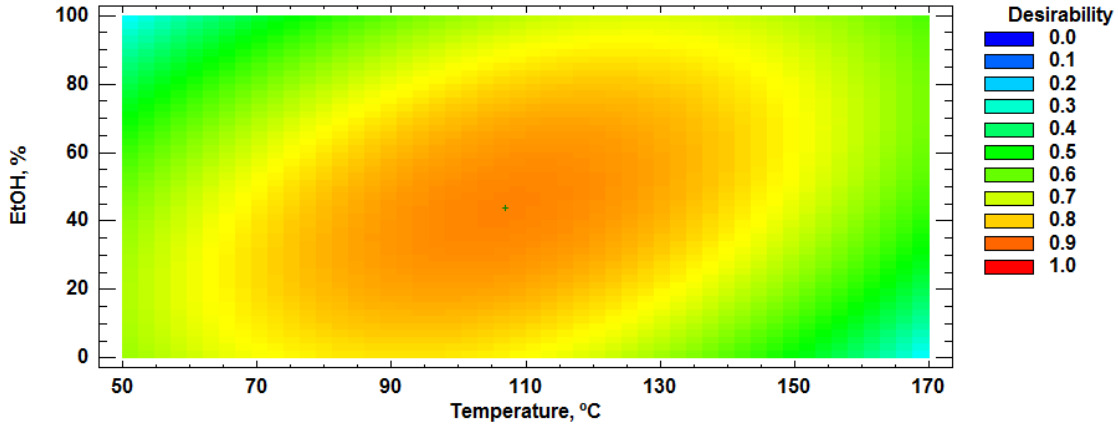

Figure 3.16. Multiple response optimization. Contours of estimated response surface

Table 3.17. Optimum pressurized liquid extraction conditions of *G. macrorrhizum* fat-free leaves. Predicted and actual values of extraction.

PLE extraction conditions			Response Variables			
	Temp, °C	Ethanol, %	Extraction yield, %	TPC, mg GAE/g[b]	EC_{50}, µg/mL[c]	TEAC, mmol TE/g[d]
Predicted	107	43	46.04[a]	378.95	3.46	8.80
Actual	107	43	39.99±0.01	376.51±0.40	3.97±0.24	7.99±0.01
		RSD[e], %	*-10.69*	*-0.46*	*+9.20*	*-7.10*

Values represented as mean ± standard deviation. [a]Extraction yield predicted value was obtained in the multiple response optimization including yield; [b]mg gallic acid equivalents/g extract; [c]DPPH• assay efficient concentration (ug/ml); [d]mmol trolox equivalents/g extract; [e]relative standard deviation

The total extraction yield obtained by SFE-CO_2 under optimal conditions (***Table 3.4***) was much lower than those obtained by PLE under optimal extraction conditions (***Table 3.17***) (3.55, 39.99 %, respectively).

3.7.7. Analysis of the phytochemical profile of *G. macrorrhizum* PLE extract

The identification of phytochemical profile of *G. macrorrhizum* leaves PLE extracts was based on characteristics of monoisotopic mass detected under ESI negative ionization mode. ***Figure 3.17*** shows UPLC-qTOF-MS/MS chromatogram. ***Table 3.18*** shows the profile of *Geranium machrorrhizu*m leaves PLE extracts. In this study, 22 compounds were tentatively identified as quinic acid derivatives, gallic acid and its derivative, theogallin, glucosyl gallate, protocatechuic acid, pyrocatechol glucuronide, 4-HBA, chlorogenic acid, rutin, quercetin and its derivatives, kaempferol and its derivatives, rosmarinic acid by comparing mass spectrometric data with the literatures and characteristic diagnostic fragment ions.

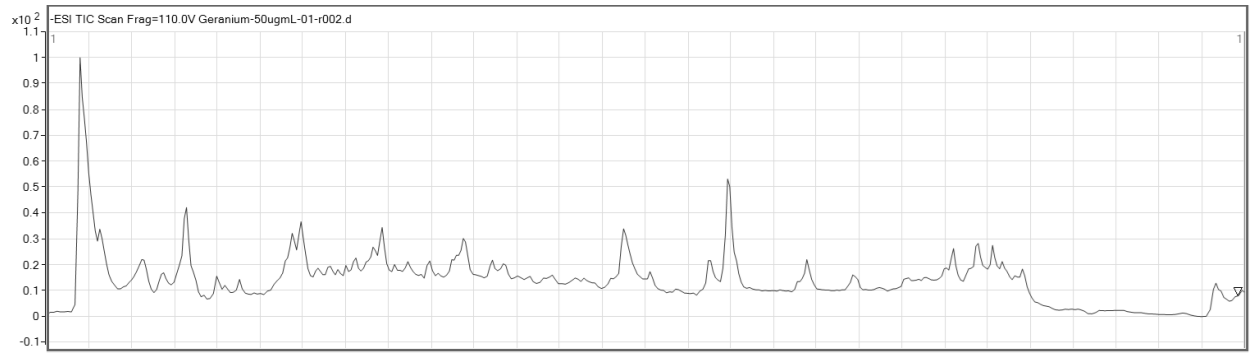

Figure 3.17. UPLC-qTOF-MS/MS chromatogram of *G. macrorrhizum* leaves PLE extract

Nataša Nastić et al. [167] screened and reported activity of non-volatile compounds from rhizomes and aerial parts of *G. macrorrhizum* subcritical water extracts. HPCL-DAD detected high concentration of Gallic acid (1512 ± 151 mg/100 g DE), lower concentration of protocatechuic acid (234 ± 23 mg/100 g DE), ferulic acid (128 ± 13 mg/100 g DE), chlorogenic acid (106.9 ± 10.7 mg/100 g DE), catechin (97.7 ± 9.8 mg/100 g DE), vanillic acid (14.3 ± 1.4 mg/100 g DE) and *p*-coumaric acid (8.64 ± 0.86 mg/100 g DE) in subcritical water extracts. Radulović et al. also analysed the phenolic composition of methanol extracts from *G. macrorrhizum* by liquid chromatography. Authors reported that extracts mostly consist of glycosylated bound phenolic compounds, mostly gallic and ferulic acid [168]. Sharopov et al. compared and reported methanol extracts from leaves and roots. Gallic acid was the major compound in both extracts. Total phenolic content was higher in methanol extract from roots (993.5 caffeic acid equivalents/1mg extract) than in leaves (753 mg caffeic acid equivalents/1 mg extract). Total flavonoid content was higher in leaves (49.0 mg quercetin equivalents/1 mg extract) than in roots (4.5 mg quercetin equivalents/1 mg extract) [169]. Miliauskas reported quercetin and its derivatives and ellagic acid in extracts from *G. macrorrhizum* leaves using the EtOH and H_2O as solvents. [14]. Taşkın *et al.* identified phenolic compounds of *Geranium purpureum* aerial parts. Authors reported caffeic acid derivative, quinic acid, galloyl-hexoside, malic acid, gallic

acid, methyl gallate hexoside, chlorogenic acid, caffeic acid, rutin, dicaffeoyl quinic acid and quercetin [170].

To my best knowledge, there are no previous reports about theogallin and glucosyl gallate presence in *Geranium* plants. According to sensory studies, theogallin, as well as gallic acid, can increase umami intensity of sodium l-glutamate [171].

Table 3.18. Tentatively identified compounds from defatted Geranium leaves PLE extract by UPLC-qTOF-MS/MS analysis

R.T. (min)	Name	Formula	Monoisotopic mass*	[M-H]- (m/z)	MS2 product ions (m/z)
0.425	Quinic acid derivative	C19H34O17	534.1796	533.1752	383.121, 191.0575
1.121	Gallic acid	C7H6O5	170.0215	169.0153	125.0255
1.734	Theogallin	C14H16O10	344.0744	343.0688	169.0134
1.848	Glucosyl gallate	C13H16O10	332.0744	331.0693	169.0144
1.860	Protocatechuic acid	C7H6O4	154.0266	153.0204	110.0316
2.091	Pyrocatechol glucuronide	C12H14O8	286.0648	285.0532	108.0235
2.433	4-HBA	C7H6O3	138.0317	137.0252	108.0221, 930.0345
2.878	Chlorogenic acid	C16H18O9	354.0951	353.0886	191.0556
2.978	Catechin	C15H14O6	290.0790	289.0720	191.0339, 247.0254
3.091	Gallic acid derivative	C20H20O14	484.0853	483.0784	271.0466, 169.0134
4.487	Rutin	C27H30O16	610.1534	609.1468	300.0276
4.957	Quercetin hexoside	C21H20O12	464.0955	463.0881	300.0274
5.168	Quercetin derivative	C21H20O11	448.1006	447.0948	227.0403, 284.0343
5.218	Kaempferol coumaroyl hexose	C27H30O15	594.1585	593.1522	285.0422
5.471	Rosmarinic acid	C18H16O8	360.0845	359.0772	-
5.588	Quercetin hexoside	C21H20O12	464.0955	463.0890	301.0358
5.813	Quercetin derivative	C27H28O15	592.1428	591.1352	301.0358
5.850	Quercetin hexoside	C21H20O12	464.0955	463.0893	301.0359
6.112	Quercetin derivative	C27H28O15	592.1428	591.1363	301.0346
6.218	Quercetin derivative	C27H26O15	590.1272	589.1208	301.0365
6.775	Quercetin	C15H10O7	302.0427	301.0353	271.0277
7.767	Kaempferol	C15H10O6	286.0477	285.0408	167.053

*Monoisotopic mass according to available literature

CONCLUSION

Nowadays phytochemicals of food plant material are highly investigated by researches. Furthermore, they are widely used in medicine. *G. macrorrhizum* and *R. carthamoides* showed to be valuable herbs, hence, they are important genus.

1. The following chemical composition were determined for *G. macrorrhizum* leaves, roots and *R. carthamoides* roots. *G. macrorrhizum* leaves had the highest nitrogen content (2.66 %), followed by roots (0.89 %) and *R. carthamoides* roots (0.56 %). Determination of fat content by Soxhlet-He extraction technique showed that fat content of plant materials ranged from 0.67 to 3.97 %. Fat content of *G. macrorrhizum* leaves were almost 2 times higher than in *G. macrorrhizum* roots and almost 6 times higher than *R. carthamoides* roots (3.97, 1.91, 0,67 %, respectively). The ash content of analyzed plants ranged from 4.97 to 15.64 %. *R. carthamoides* roots showed the highest ash content (15.64 %). Water content all all analyzed materials ranged from 6.80 to 12.00 %. *G. macrorrhizum* roots had the highest water content (12.00 %), followed by roots and *R. carthamoides* (12.00, 6.80 %, resepectively).

2. The effect of different parameters, pressure, temperature and time on the supercritical carbon dioxide extraction yield assisted on *G. macrorrhizum* leaves was investigated. The determined optimal SFE-CO_2 extraction conditions for *G. macrorrhizum* leaves were 462 bars, 55 °C and 52 min, yielding 3.35 % on the laboratory scale extractor. Under the same conditions the yield of *G. macrorrhizum* leaves on the pilot scale extractor was 3.15 % (medium PS) and 3.41 % (large PS), following by roots and *R. carthamoides* roots 1.20 and 0.74 %, respectively.

3. The total phenolic content ranged from 0.21 to 184.12 mg GAE/g DW. The highest total phenolic content was obtained in *G. macrorrhizum* leaves initial plant material (184.12 mg GAE/g). The free radical scavenging capacity ranged from 0.56 to 1011.53 mg TE/g DW for $ABTS^{\bullet+}$ assay, from 0.11 to 496.67 mg TE/g DW for $DPPH^{\bullet}$ assay and from 1.24 to 59.33 mg TE/g DW for ORAC assay. SFE-CO_2 extract of *G. macrorrhizum* leaves under optimal conditions had the highest total phenolic content (2.66 mg GAE/g DW) among all the three plant extracts under the same optimal conditions. The radical scavenging capacity of the starting plant materials decreased in the following order: *G. macrorrhizum* roots > *G. macrorrhizum* leaves > *R. carthamoides* roots.

4. GCxGC/ TOF MS method was applied for the volatile compounds determination in *Geranium macrhorrhizum* SFE-CO_2 optimal extract. It showed to be constituted mainly by waxes. The volatile compounds namely, trans-β-elemenone (2.58 %), α-curcumene (0.35 %), germacrone (0.13 %), γ-elemene (0.12 %) were found to be the sesquiterpenes of *G. macrorrhizum* leaves SFE-

CO_2 extract. The germacrone under various SFE-CO_2 extraction conditions ranged from 4.8 to 32.1 μg/mg.

5. UPLC/ESI-QTOF-MS analysis did indicate the presence of several compounds in SFE-CO_2 extracts of *G. macrorrhizum* leaves, roots and *R. carthamoides* roots. Chromatographic analysis showed 6 compounds in *G. macrorrhizum* leaves, 4 in roots and 1 *R. carthamoides* roots which were tentatively identified.

6. The total sum of polar (hexane) and non-polar (acetone, ethanol) fractions of different plant materials was: 8.89 % of *G.macrorrhizum* leaves, 27,27 % of *G.macrorrhizum* roots and 5,44 % of *R.carthamoides* roots. The yield of hexane fraction ranged from 0.37 to 2.92 %; the yield of acetone fraction from 1.39 to 9.58 %; the yield of ethanol fraction from 3.68 to 16.69 %. The highest soluble parts were obtained using acetone as a solvent. Yield of extract of *G. macrorrhizum* leaves and roots obtained by Soxhlet extraction technique using hexane was higher than those obtained by SFE-CO_2. SFE-CO_2 extraction yields were higher than SLE-He extraction yields.

7. It was the first report on the bio-refinery study of *G. macrorrhizum* leaves. The effect of different parameters, temperature and the ratio of ethanol in the extracting solvent on the pressurized liquid extraction assisted on *G. macrorrhizum* defatted leaves was investigated. The condition of extraction was optimized using the response surface methodology to maximize the yield, total phenolic content and antioxidants activity. Extraction yield was removed for multiple response optimization. The following optimum extraction conditions were suggested by the model: 107 ºC temperature and 43 % of ethanol in the solvent mixture. A good correlation between TPC and antioxidant activity of extracts was observed. Extract obtained under optimal conditions showed high TPC content (378.95 mg GAE/g) and antioxidant activity ($DPHH^{\bullet}$ (EC_{50}) 3.46 μg/mL, $ABTS^{\bullet+}$ (TEAC) 8.80 mmol TE/g). These results are supporting the idea that phenolic compounds act as contributors of the antioxidant activity in plant material.

8. UPLC/ESI-QTOF-MS/MS method was applied for the determination of phenolic compounds from *Geranium macrhorrhizum* PLE extract under optimal conditions. Results showed that the PLE extract under optimal conditions was mainly constituted by phenolic acids (such as gallic acid, protocatechuic acid, chlorogenic acid) and flavonols (such as catechin).

The results obtained in this work demonstrated the potential of *G. macrorrhizum* and *R. carthamoides* for bio-refinery in order to obtain high-value products. It would improve process both economically and environmentally. From pharmaceutical point of view, both genus could be considered as valuable plants due to their potent bioactivity.

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