KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

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Processing of oregano (*Origanum vulgare L.*) into aroma compound and antioxidant fractions by conventional and high pressure extraction techniques

Master‘s thesis in Food Science and Safety

Supervisor
Lect. dr. Vaida Kitrytė

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Paprastojo raudonėlio (*Origanum vulgare L.*) perdirbimas į aromato junginių ir antioksidantų frakcijas tradiciniais ir aukšto slėgio ekstrakcijos metodais

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DECLARATION OF ACADEMIC INTEGRITY

05 June 2017
Kaunas

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SANTRAUKA

Paprastasis raudonėlis (*Origanum vulgare* L.) yra daugiametis, žydintis augalas, plačiai auginamas Europoje, Azijoje ir Amerikoje. Raudonėlis ir jo produktai (prieskoniai, ekstraktai, eteriniai aliejai ir t.t) dėl savo antiseptinių, antioksidacinių, imunitetą stiprinančių savybių yra plačiai pritaikomi kulinarijos, farmacijos ir kosmetikos produktuose. Todėl, šio baigiamojo magistro projekto tikslas buvo išskirti iš paprastojo raudonėlio (Origanum vulgare L), užauginto Lietuvoje, aromato junginių ir antioksidantų frakcijas, panaudojant tradicinius ir kelių pakopų aukšto slėgio ekstrakcijos metodus.

Siekiant įgyvendinti šį tikslą, lakiųjų ir skirtingo poliškumo nelakiųjų frakcijų išskyrimui, buvo pritaikytos skirtingos tradicinės ir aukšto slėgio ekstrakcijos: hidrodistiliacija, Soksleto ekstrakcija, kietos fazės-skysčių ekstrakcija, superkrizinė ekstrakcija anglies dioksidu, padidinto slėgio tirpikliais ekstrakcija. Raudonėliui buvo pritaikyta kelių pakopų aukšto slėgio frakcionavimo schema ir optimalūs superkrizinės CO2 ekstrakcijos ir ekstrakcijos padidintame slėgyje tirpikliais parametrai (slėgis, temperatūra, laikas, modifikatoriaus priedas) buvo nustatyti siekiant išgauti didžiausias polinių ir nepolinių frakcijų išeigas su didžiausiu bendru fenolinių junginių kiekiu, panaudojant skirtingus tirpiklius. Be to, šių ekstrakcijų efektyvumas buvo palygintas su tradiciniais ekstrakcijos metodais. Prieš ekstrakcijas, raudonėlio žaliavoje buvo nustatytas riebalų kiekis, baltymų kiekis, mineralinių medžiagų kiekis, drėgmė, *in vitro* bendras fenolinių junginių kiekis, radikalų sujungimo geba ir eterinių aliejų lakiųjų junginių kompozicija. Skirtingo poliškumo ekstraktai ir kietas likutis po kiekvienos ekstrakcijos pakopos buvo analizuojami, įvertinant *in vitro* antioksidacinį potencialą Folin-Ciocalteu‘s, $DPPH^{\bullet}$, $ABTS^{\bullet+}$ ir ORAC metodais. Ekstraktų priedas buvo panaudotas rapsų aliejuje, siekiant padidinti jo oksidacinį stabilumą. Ekstaktuose taip pat buvo nustatyta riebalų rūgščių sudėtis (GC-FID), fitocheminė kompozicija (UPLC-QTOF-MS), taip pat skvaleno kiekis HPLC metodu.

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Research area and field: Technological Sciences, Food Technology

Key words: *Origanum vulgare, essential oils, conventional extraction methods, antioxidant activity, squalene, fatty acids composition.*

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SUMMARY

Oregano (*Origanum vulgare L*.) is a perennial flowering plant, widely cultivated in Europe, Asia and America. Oregano is used as a spice in culinary, it is also used in folk medicine for its antiseptic, antioxidant, toning, immune-enhancing properties. For important aroma compounds presence in oregano, oregano essential oil has wide application in cosmetics and personal hygiene products. This research was aimed to process oregano (*Origanum vulgare* L.), grown in Lithuania, into aroma compound and antioxidant fractions by conventional and multi-step high pressure extraction techniques.

In order to achieve this goal there were carried out different extractions: hydrodistillation, Soxhlet extraction, solid-liquid extraction (conventional extraction techniques) and supercritical fluid extraction with carbon dioxide, pressurized liquid extraction (high pressure extraction techniques). Solid residues after supercritical fluid extraction with carbon dioxide has been applied for the pressurized liquid extraction (using response surface methodology for pressurized liquid extraction with acetone was performed optimization of extraction time and pressure, which produces the highest extract yields). After optimization, the solid residues further were utilized by pressurized liquid extraction with different solvents.

Total extract phenolics determined using the Folin-Ciocalteu's method, antioxidant activity was measured using $DPPH^{\bullet}$, $ABTS^{+}$ scavenging assays. Oxygen radicals absorbance capacity was measured by ORAC assay. Solid residues antioxidant activity was measured with the same *in vitro* antioxidant activity methods by approaching QUENCHER procedure. Selected extracts oxidative stability in rapeseed oil was measured with oksipres method.

Phytochemical characterization of chosen extracts were assessed: preliminary composition was identified by UPLC-QTOF-MS, fatty acids composition was determined by GC-FID method, squalene amount in different extracts was evaluated by HPLC analysis.

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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
$ABTS^{\bullet+}$	2,2′-azino-di-3-ethyl benzothiazoline-6-sulfonic acid
ANOVA	one-way analysis of the variance
CCD	central composite design
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl
DW	dry weight
EAE	enzyme assisted extraction
Fluorescein	2-(3-hydroxy-6-oxo-xanthen-9-yl) benzoic acid
GAE	gallic acid equivalents
GC/MS	gas chromatography/mass spectrometry
HD	hydrodistillation
HPLC	high-performance liquid chromatography
IP	induction period
MAE	microwave assisted extraction
ORAC	oxygen radical absorption capacity
PBS	phosphate buffered saline
PLE	pressurized liquid extraction
RSM	response surface methodology
R. T	room temperature
SFE	supercritical fluid extraction
SLE	solid-liquid extraction
TE	trolox equivalents
TPC	total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UAE	ultrasound assisted extraction
UPLC/ESI-QTOF-MS	ultra-performance liquid chromatography quadrupole time of flight mass spectrometry

INTRODUCTION

Oregano (*Origanum vulgare L.*) is a flowering herb, widespread in Mediterranean, Baltic and Nordic countries [0]. Oregano is rich natural resource of various bioactive constituents and is used for both traditional and modern medicines, nutraceuticals, food supplements, and as well as flavors and fragrances in culinary and cosmetic industry. The main bioactive compounds of oregano plants are volatile terpenes and terpenoids (important constituents of essential oils) and non-volatile phenolic compounds with multiple biological activities, such as antioxidant, antimicrobial, fungicidal, anti-inflammatory and immune-strengthening [2]. The qualitative and quantitative composition of these bioactive constituents may significantly vary due to the plant subspiecies and chemotype, growing region and climate conditions, as well as harvest time and sample preparation for analysis (for example, particle size reduction) [3,4,5]. The first step in the processing of medicinal and aromatic plants into higher-added value ingredients is the production of herbal extracts and essential oils, using a variety of methods, varying from simple traditional technologies to advanced extraction techniques and allowing broad range and/or selective separation of soluble plant metabolites from plant tissues and insoluble cellular marc [0]. Extraction of compounds from plant tissues has usually been accomplished by conventional extraction processes (e.g., hydrodistillation, Soxhlet extraction, solid-liquid extraction). Conventional extraction techniques are time-consuming (e.g., 2-6 hours for hydrodistillation, 4-12 hours for Soxhlet extraction, 2-24 hours for maceration or solid-liquid extraction) and requires large amounts of solvents. Also, commonly used solvents, such as hexane and methanol, has limited applications in food and pharmaceutical industries and due to the particular toxicity has regulated maximum residue limits in the extracted foodstuff or food ingredient, for example, hexane – 1-30 mg/kg (depending on product type), methanol – 10 mg/kg [6]. Important factors to consider are the properties of plant material as well. For example, oregano grown in Mediterranean region are rich in essential oils, while those cultivars from colder climate zones has low essential oils yield, therefore are less used for hydrodistillation, but could be successfully utilized for non-volatile bioactive compound, e.g. antioxidant rich-fractions, recovery.

So far as the conventional solvent extraction may recover target constituents only partially and also large portion of the bioactive constituents may be remained in solid residues after extractions, further processing steps are necessary for increasing valorization effectiveness. High pressure extraction techniques, such as supercritical fluid extraction with carbon dioxide (SFE-CO_2) and pressurized liquid extraction (PLE) has a gained a lot of attention as alternative and efficient extraction techniques for bioactive compounds isolation. The main advantages of SFE-CO_2 is utilization of nontoxic, nonflammable, inexpensive solvent for extraction, as well as the

ability to modify process conditions (pressure, time, temperature, co-solvent etc.), yielding high purity extracts, enriched with bioactive compounds of interest. Similarly, PLE provides the ability to extract different polarity fractions, efficiently using food-grade solvents (ethanol or water) during the remarkably shorter extraction time, as compared to the corresponding conventional extraction techniques. Recently it was shown that multi-step high pressure fractionation of various plant material might be a promising strategy for various plant material and food industry by-product valorisation and conversion into functional ingredients. At the first step, SFE-CO_2 is recommended for lipophilic fraction isolation as an alternative technique to conventional organic solvent extraction [5]. Afterwards polar components, e.g. polyphenols with potential antioxidant capacity *in vitro* and *in vivo*, can be extracted from the SFE-CO_2 residue via PLE consequently using food-grade solvents. Application of response surface methodology (RSM) combining mathematical and statistical techniques for modelling and analyzing the process and determining the effect of the independent variables for selected responses would be an important step in optimizing the extraction of oregano in the production of high added value functional ingredients. The optimization of extraction conditions for target phytochemical isolation applying this fractionation concept was previously reported for *B.crassifolia* roots and leaves [7], blackcurrant buds [8], raspberry pomace [9], chokeberry pomace [10], wheat and rye bran [11], [12], and amaranth [13,14]. Looking at the data in scientific literature, high pressure extraction (subcritical water, CO_2 and PLE with methanol) was previously employed to recover antioxidatively-active phenolic compound fractions [3] and valuable flavonoids [4] from oregano as well. For these purposes, several extraction conditions were tested and reported as optimal in order to obtain the highest amounts of target constituents. Nevertheless, these studies are mainly lacking the systematic multi-step valorization approach, since in all cases only one step high-pressure fractionation (e.g., SFE-CO_2 or PLE) is performed and only the selected phytochemical indices of the obtained extracts are reported. Furthermore, to the best of our knowledge, solid plant material residues after extraction, which may contain a considerable portion of cell wall-bound bioactive constituents, are not further investigated and utilized. In addition, there is a lack of data comprehensively comparing the efficiency (extraction yield, time, etc.) of the aroma compound and antioxidant extraction from oregano via conventional and high-pressure extraction techniques.

Therefore, this research was aimed to process oregano (*Origanum vulgare* L.), grown in Lithuania, into aroma compound and antioxidant fractions by conventional and multi-step high pressure extraction techniques. To achieve this aim, the following objectives were raised:

1. To characterize oregano by determining the selected chemical composition (lipid content, protein content, mineral content, moisture) and *in vitro* antioxidant activity (total phenolic content and radical scavenging capacity) indices.

2. To characterize oregano by determining its chemotype according to the volatile compound profile and to evaluate the impact of particle size reduction on essential oil yield and composition.
3. To develop multi-step high pressure fractionation scheme of oregano by determining optimal SFE-CO_2 and PLE parameters (pressure, temperature, time, co-solvent addition) in order to obtain the highest non-polar and polar fraction yields with highest total phenolic content.
4. To compare the efficiency of conventional and high-pressure extraction techniques for the target fraction recovery from oregano.
5. To evaluate *in vitro* antioxidant potential and of various oregano non-polar and polar extracts and solid residues after each step of extraction.
6. To evaluate possibilities of the selected oregano extracts to increase the oxidative stability of rapeseed oil.
7. To evaluate phytochemical composition of various oregano non-polar and polar extracts, applying chromatographic and mass spectrometric methods.

1. LITERATURE REVIEW

1.1. Oregano (*Origanum vulgare* L.): morphology, chemical composition and cultivation

Oregano (*Origanum vulgare L.*) is flowering herb, which belongs to *Lamiaceae* family plants. The *Lamiaceae,* also called the mint family, is one of the most widespread plant families, comprised of about 236 genera and 6900-7200 plant species. The largest genera are *Salvia, Scrutellaria, Stachys, Vitex and Thymus.* Many plants of this family are known as highly aromatic in all anatomical parts and include many widely used culinary herbs, such as basil, mint, rosemary, marjoram, sage, thyme, oregano [15]. Based on morphological criteria, the genus *Origanum* of *Lamiaceae* family has been classified into 3 groups, 10 sections, 38 species, 6 subspecies and 17 hybrids [16]. There are four oregano groups, commonly used for culinary purposes: Greek oregano (*Origanum vulgare subsp hirtus (Link) letswaart*), Spanish oregano (*Coridohymus capitatu (L) Hoffmanns and Links*), Turkish oregano (*Origanum onites L*), and Mexican oregano (*Lippia graveolens Kunth or Lippia berlandieri)* [17].

Oregano has erect, hairy, square-formed stem, and grows up to 90 cm. Its flowers are purple or white (**Fig. 1**) and are pollinated by insects. They are tubular and two-lipped, have four protruding stamens. The calyx (inside and outside) is hairy. Blooming period is from June to September [18]. It is found that oregano, which grows at high altitudes (1760 m), are shorter than those growing at low altitude (200 m). Plant shortening at high altitude is associated with the shorter growing period, reduced temperatures, water and nutrient limitations. It is also reported that plant leaves are larger and thicker at mid- and high altitudes [19].

Fig. 1 Oregano plant (adapted from Amédée Mascle, Atlas des plantes de France [20])

O. Vulgare is a plant native to the Mediterranean regions, also common in the Baltic and Nordic countries. The composition and amount of the secondary metabolites of these plants depend on climatic factors, altitude, harvest time, and its state of growth [17]. Therefore, the study of these factors and their influence on their cultivation is important for their better utilization both at

domestic and industrial levels. For example. *O. vulgare* plants from the Mediterranean climate zone are very rich in essential oil, whereas those originating from the continental climate zone have less gland hairs and are essential oil-deficient. In addition, chemical composition of essential oil (qualitative and quantitative composition of volatile compounds) depends on the place of its distribution and at which growing stage plant was harvested [21]. Due to the different cultivation conditions, increase or decrease in amounts of various endogenous plant constituents may be observed. Typically, oregano plants prefer well-drained soils, which are neutral or alkaline, also sunny areas have a positive impact on growth. It is also well preserved during winter periods [18]. In Lithuania, the oregano population mostly concentrated to Southeastern and Eastern Lithuania. The largest areas were found in Trakai, Berzgainiai Reserve, Venta Regional Park and Panemuniai Regional Park (**Fig. 2**) [18].

Fig. 2 Oregano distribution in Baltic countries (adapted from Nordic gene bank [18])

Oregano contains an impressive list of plant-derived chemical compounds that are known to have disease preventing and health promoting properties [22]. Oregano and other *Lamiaceae* family plants nutritional value is compared in **Table 1** [23]. These plants are popular and widely used as culinary herbs, also widely used as various researches objects. Carbohydrates amount reaches about 64%, proteins – 11%, fat – 10%. Carbohydrates amount is quite similar in different presented *Lamiaceae* plants. Protein content also is similar in three plants, only rosemary has much lower amount. Fat content differs in basil plant. Oregano herb is a good source of carotenoids. Carotenoids such as β-carotene, lutein-zeaxanthin, β-crypto-xanthin were found in oregano.

Table 1. Chemical composition and nutritional value of the selected *Lamiaceae* family plants

Components	Chemical composition (100 g of plant material)			
	Oregano (*Origanum vulgare*)	Basil (*Ocimum basilicum)*	Rosemary (*Rosmarinus officinalis*)	Sage (*Salvia officinalis*)
Carbohydrates	64.4 g	61 g	64.1 g	60.7 g
Sugars	4.1 g	1.7 g	-	1.7 g
Protein	11 g	14.4 g	4.9 g	10.6 g
Total Fat:	10.3 g	4 g	15.2 g	12.7 g
Saturated fat	-	0.2 g	7.4 g	7 g
Monounsaturated fat	0.7 g	0.5 g	3 g	1.9 g
Polyunsaturated fat	5.2 g	2.2 g	2.3 g	1.8 g
Total Omega-3 fatty acids	4180 mg	1509 mg	1076 mg	1230 mg
Total Omega-6 fatty acids	1050 mg	659 mg	1160 mg	530 mg
Dietary Fiber	42.8 g	40.5 g	42.6 g	40.3 g
Phytosterols	203 mg	106 mg	58 mg	244 mg
Vitamins:				
Folates	274 µg	274 µg	307 µg	274 µg
Niacin	6.22 mg	6.9 mg	1 mg	5.7 mg
Pantothenic acid	0.92 mg	-	-	-
Pyridoxine	1.21 mg	-	-	-
Riboflavin	0.32 mg	0.3 mg	0.4 mg	0.3 mg
Thiamin	0.34 mg	0.1 mg	0.5 mg	0.8 mg
Vitamin A	6903 IU*	9376 IU	3128 IU	5900 IU
Vitamin C	50 mg	61.2 mg	61.2 mg	32.4 mg
Vitamin E (alpha tocopherol)	18.9 mg	7.5 mg	-	7.5 mg
Vitamin K	621.7 µg	1715 µg	-	1715 µg
Vitamin B6	1.2 mg	2.3 mg	1.7 mg	2.7 mg
Choline	32.3 mg	54.9 mg	-	43.6 mg
Betaine	9.8 mg	16.1 mg	-	-
Minerals:				
Sodium	15 mg	34 mg	50 mg	11 mg
Potassium	1669 mg	3433 mg	955 mg	1070 mg
Calcium	1576 mg	2113 mg	1280 mg	1652 mg
Copper	0.94 mg	1.4 mg	0.5 mg	0.8 mg
Iron	44 mg	42 mg	29.2 mg	28.1 mg
Magnesium	270 mg	422 mg	220 mg	428 mg
Manganese	4.67 mg	3.2 mg	1.9 mg	3.1 mg
Zinc	4.43 mg	5.8 mg	3.2 mg	4.7 mg
Phosphorus	200 mg	490 mg	70 mg	91 mg
Selenium	5.9 µg	2.8 µg	4.6 µg	3.7 µg
Essential oils	0.8-3.2% [24,25]	0.05-0.8% [26,27]	0.3-2.5% [28,29]	0.4-2.5% [30]

Carotenoids are thought to provide health benefits in decreasing the risk of cancers and eye disease β-carotene is beneficial due its ability to be converted to vitamin A, lutein and zeaxanthin may be protective in eye disease because they absorb damaging blue light that enters the eyeOregano herb also presents huge amounts of vitamins and minerals (**Table 1**) [23]. As could

be seen from **Table 1** potassium amount differences in different plants are obvious, manganese and copper amounts differs, but not so much. Potassium is an important component of cell and body fluids that helps control heart rate and blood pressure caused by high sodium. Manganese and copper are utilized by the body as co-factors for the antioxidant enzyme, superoxide dismutase. Iron helps prevent anemia. Magnesium and calcium are important minerals for bone metabolism [31].

All endogenous oregano constituents (**Table 1**) can be divided into two broad groups. One group is comprised of primary metabolites, which are the chemical substances aimed at growth and development, such as carbohydrates, amino acids, proteins and lipids. Another group is secondary plant metabolites, which are synthesized as a part of their defense system against diseases and herbivores in order to increase plant overall ability to survive under the particular conditions. Secondary plant metabolites can be found in various anatomical parts (roots, leaves, seeds, fruits, flowers) and some of them possess particular bioactive properties. Bioactive compounds of oregano plants are divided into three main categories: volatile terpenes and terpenoids (important constituents of essential oils), non-volatile alkaloids and phenolic compounds with multiple biological activities, which represent the largest group of secondary metabolites in plants of *Lamiaceae* family [32,33,34].

1.2. Bioactive volatile constituents of oregano

1.2.1. Biosynthesis of essential oils

Oregano essential oils (EO), composed of various volatile compounds, are mostly presented in leaves, but also could be found in all parts of the plants. It is valuable ingredient in cosmetic, flavoring, fragrance, perfumery, pesticide, and pharmaceutical industries. In an essential oil, there can be alicyclic and aromatic hydrocarbons, as well as their oxygenated derivatives (alcohols, aldehydes, ketones and esters), sulfur and nitrogenous substances. Studies indicated that more than 50% of oregano oil consist terpene compounds such as carvacrol and thymol [17]

Mostly, the essential oil compounds belong to various terpenes classes. Terpenes include more than 40 000 compounds [35]. All terpenes are constructed from two types of five-carbon molecules: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) [36]. Sequential condensation of IPP and DMAPP leads to the formation of prenyl diphosphates, the precursors for the most of terpenes (**Fig. 3**): geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), geranylgeranyl diphosphate (GGPP, C_{20}), and geranylfarnesyl diphosphate (GFPP, C_{25}). GPP is the precursor of monoterpenes (C_{10}) and FPP is the precursor of sesquiterpenes (C_{15}), while GGPP is the precursor of diterpenes (C_{20}) [37]. Sequential condensation of FPP and GGPP results in precursor of triterpenes (C_{30}). As depicted in Fig.3, in

aromatic plants species, biosynthesis of essential oil component occurs through two complex biochemical pathways, involving different enzymatic reactions. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the universal precursors of terpenes biosynthesis and are produced by the cytosolic enzymatic MVA (mevalonic acid) pathway or by plastidic and enzymatic 1-deoxy-D-xylolose-5-phosphate (DXP) pathway, also called the 2-Cmethylerythritol-4-phosphate (MEP) pathway [38]. Essential oils are final terpenoid products and are formed by a huge group of enzymes known as terpene synthases (TPS) [39]. In the MVA pathway, IPP is generated by decarboxylation from mevalonate-5-diphosphate by mevalonate diphosphate decarboxylase (MVD or MDD) [40]. In the MEP pathway, IPP is generated from 4-hydroxy-3-methylbut-2-enyl-diphosphate (HMBPP) by HMBPP reductase [41]. In the MEP pathway, both IPP and DMAPP can be produced by HMBPP reductase [42]. In the MVA pathway, IPP is converted into DMAPP by IPP isomerase (IDI) [43], which also operates in plastids and mitochondria [44].

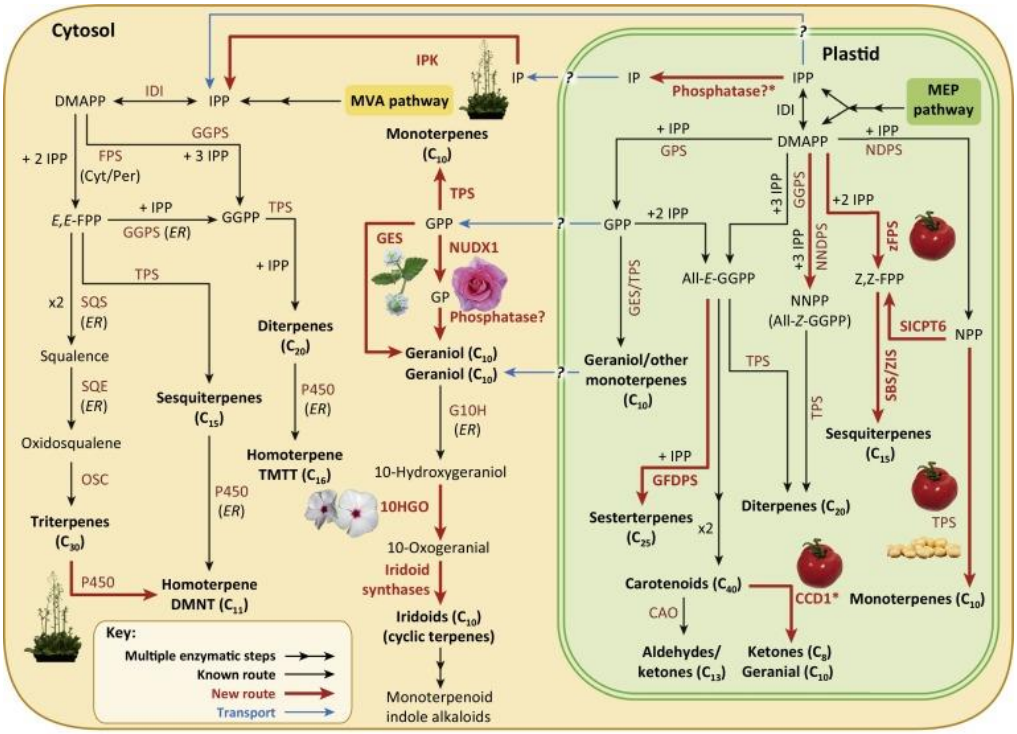

Fig. 3 Biosynthesis of terpenes (adapted from Sun et. al [45])

There are a lot of factors affecting the quality of oregano essential oil. Number of researchers report that the great variability in the chemical composition of essential oils is mainly occurring due to the origin of the material rather than the influence of the environment (climate and cultivation conditions) [46]. However, other scientists give a more preponderant role to the environment, especially in one plant distance from another, season of the year when plant is harvested and the amount of water used in the irrigation, or even the amount of artificial or natural light used in growing process. For example, nitrogen fertilization increased the percentage of the

main essential oil compounds thymol and carvacrol and decrease of γ-terpinene and p-cymene in *Origanum syriacum* [21].

1.2.2. Chemical composition and bioactivity of essential oils

The composition of *O. vulgare* essential oil from different geographical origins has been characterized by several authors, with carvacrol and thymol as the major components, though the proportions vary widely (**Table 2**). Also in other *Origanum* plants species important essential oil components, such as p-cymene, γ-terpinene, caryophyllene, spathulenol, and germacrene-D were reported.

Table 2. The composition of *O. vulgare* essential oil from different geographical origins

Origin	Extraction method	Detection method	Compounds	Ref.
Portugal	4 h hydrodistillation	GS-MS, GS-FID	Thymol (32.6%); γ -terpinene(25.9%); p-cymene (10.7%); β-caryophyllene (4.5%)	[47]
India	2 h steam distillation	GS-MS, GS-FID	Thymol (0–82.0%); carvacrol (27.4%); germacrene D (trace–13.3%); β-caryophyllene (0.4–8.8%)	[48]
Poland	Hydrodistillation (Deryng)	GS-MS	Carvacrol (3.6–9.1 g kg−1); thymol (2.14–8.44 g kg−1); γ -terpinene (1.5–4.9 g kg−1)	[49]
Greece	3 h steam distillation	GS-MS, GS-FID	Carvacrol (88.7%); p-cymene (3.4%); γ -terpinene (3.2%); β-caryophyllene (1.1%)	[50]
Argentina	2 h hydrodistillation	GS-MS, GS-FID	Thymol (20.5–26.1%); trans-sabinene hydrate (27.8 32.5%); γ -terpinene (5.4–15.5%); terpinen-4-ol (3.5–5.0%)	[51]
Croatia	3 h hydrodistillation	GC-MS	Thymol (40.4%); carvacrol (24.8%); p-cymene (16.8%)	[52]
Italy	2 h hydrodistillation	GC-MS	Carvacrol (54.7%); thymol (22.1%); γ -terpinene (6.0%); p-cymene (5.5%)	[53]
Turkey	3 h hydrodistillation	GC-MS	Caryophyllene (14.4%); spathulenol (11.6%); germacrene-D (8.1%); α-terpineol (7.5%); caryophyllene oxide (5.8%)	[54]

The differences in the chemical composition of *O. vulgare* essential oil may be related to distinct environmental and climatic conditions, seasonal sampling periods, geographic origins, plant populations, vegetative plant phases, and extraction and quantification methods. As given in **Table 2**, qualitative and quantitative composition of essential oils are mainly determined by means

of GC-FID and GC-MS analysis. Different parts of oregano have different essential oil composition and quantity. As reported in **Table 3**, one of the main compounds – carvacrol vary from 3.3% in root oil to 30.7 in leaf-flower oil. As well as other main constituent – thymol varies in the same tendency: from 1.1% (in root oil) to 18.8% leaf-flower oil. Also, it could be seen that in stem and root oil were identified fatty acids, while in leaf-flower oil it was not.

Table 3. Essential oil composition of different oregano parts [55]

Leaf-flower oil	**Stem oil**	**Root oil**
37 compounds	11 compounds	29 compounds
98.78%	99.51%	98.97%
carvacrol (30.73%)	palmitic acid (60.18%)	palmitic acid (58.23%)
thymol (18.81%)	linoleic acid (14.25%)	linoleic acid (12.11%)
P-cymene (10.88%)	carvacrol (6.02%)	linolenic acid (3.66%)
caryophyllene (7.73%)	thymol (3.46%)	carvacrol (3.27%)
3-carene (4.06%)	oleic acid (5.65%)	thymol (1.08%)

Several researchers confirm the antioxidant potential of essential oils from different varieties of oregano (*O. vulgare, O. compactum, O. majorana*) too [56]. Carvacrol, thymol, γ -terpinene, and linalool are known to possess strong antioxidant properties, and exhibit antibacterial activity against several bacteria [57]. Oregano essential oils has been tested as antioxidant ingredient in different kinds of food, such as fried salted peanuts, olive oil, coated peanuts [58,59]. It was reported that this essential oil increased the oxidative stability of thigh meat during frozen storage [60]. Also, it has been observed that different chemical composition of oregano essential oil produced different antioxidant activity. Refined sunflower oil was enriched with different oregano fractions obtained after molecular distillation, their composition was different as well as antioxidant activity [61].

There are multiple studies on the antimicrobial activity of essential oils and individual this fraction constituents from different types of oregano. Essential oils of species of the genus *Origanum* have been found to exhibit activity against gram negative bacteria such as *Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae, Yersinia enterocolitica* and *Enterobacter cloacae*, and gram-positive ones such as *Staphylococcus aureus, Staphylococcus epidermidis, Listeria monocytogenes* and *Bacillus subtilis* [62,63]. They also have antifungal capacity against *Candida albicans, C.tropicalis, Torulopsis glabrata, Aspergillus Niger, Geotrichum and Rhodotorula*; but not against *Pseudomonas aeruginosa* [64]. The antimicrobial activity of the isolated components, as well as that of the essential oil, has been evaluated. Carvacrol and thymol have the highest levels of activity against gram negative microorganisms active. The minimum inhibitory concentration values for essential oils have been established between 0.28-1.27 mg / ml for bacteria and 0.65-1.27 mg / ml for fungi [62]. The ethanolic extract of a clonal line of oregano

inhibited the action of *Listeria monocytogenes* in broth and other meat products [65]. It has also been found that the essential oil of oregano is very valuable in the inhibition of *E. coli O157:H7* [66]. In addition, some animal models for cancer have shown that several monoterpenes possess different properties anticarcinogenic acting at different molecular and cellular levels. These studies have important implications for the food, cosmetics and pharmaceutical industries.

1.2.3. Isolation of essential oils by conventional and high pressure extraction techniques

The conventional methods, widely used to retrieve essential oils from plant material, are hydrodistillation and simultaneous Likens-Nickerson (**Fig. 4**) extraction/distillation with organic solvents. As exemplified in **Table 4**, hydrodistilliation is most commonly applied for extraction and determination of volatile compounds from oregano of different origins. There are three types of hydrodistillation: water distillation, water and steam distillation and direct steam distillation. Hydrodistillation is usually carried out using a Clevenger apparatus (**Fig. 5**) and involves these physicochemical processes: hydrodiffusion, hydrolysis and decomposition by heat. During hydrodiffusion, a part of volatile essential oil constituents dissolves in the water, and due to the osmosis this oil-water solution permeates through the swollen plant membranes and finally reaches the outer surface, where the oil is vaporized by the passing steam. The vapor mixture of water and essential oil constituents is condensed by indirect cooling with water. From the condenser, distillate flows into a separator, where oil separates automatically from the aqueous phase [67,68]. Hydrolysis is defined as a chemical reaction between water and certain constituents of essential oils. Esters, in the presence of water, especially at high temperatures tend to react with water to form acids and alcohols. Almost all constituents of essential oils are unstable at high temperature. Temperature in steam distillation is established by operating pressure, while in water distillation and in water and steam distillation the operating pressure is usually atmospheric. In order to choose the appropriate hydrodistillation technique, is important to know that a portion of highly volatile component, as well as water-soluble components, can be lost during the extraction process. In case of water distillation, the material is completely immersed in water, leading to the following disadvantages of this method: esters are sensitive to hydrolysis, monoterpene hydrocarbons and aldehydes are susceptible to polymerization, while terpeno-phenols tend to dissolve in still water, so their removal is not full. In water and steam distillation, the oil yield is higher and the components are less susceptible to hydrolysis and polymerization, however as for solely water distillation, it is a time-consuming process, also high boiling temperature can lead to the undesirable modifications in qualitative and quantitative composition of volatiles. After hydrodistillation, not only essential oils are obtained, but also and water-soluble hydrolats, which are commonly used in cosmetics industry. However, due to the particular antioxidant properties, this

fraction could also find potential applications in food industry, mainly as a source of water-soluble natural antioxidants and novel functional ingredients [67,68].

In Likens-Nickerson extraction process the extracting solvent should be low boiling and immiscible in apparatus, less dense than water (pentane, diethyl ether). This extraction is carried out at ambient or slightly reduced pressure. Likens-Nickerson apparatus first reported was in 1964 by Likens and Nickerson. The glassware constructed that both solvents could return to their starting vessels. Upon heating, volatile compounds in the steam are transferred to the solvent and both liquids condense. After extraction, extracts are collected and dried using anhydrous sodium sulfate or by freezing and decanting solvent from ice [68]. Advantages: low solvent volumes, ability to minimize thermal degradation [69]. After all above mentioned extractions, for the most part solid residues are concluded as waists, yet it could be utilized much more effective, because after extraction of essential oils in used plant material still remains high amount of other bioactive compounds, which could be used for further extractions to isolate them. In **Table 4** is presented essential oil yields obtained by different authors at different hydrodistillation conditions.

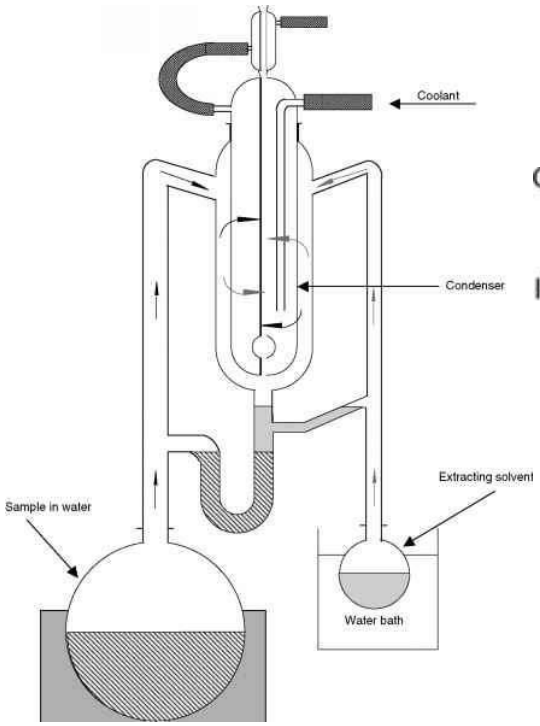

Fig. 4 Likens-Nickerson apparatus

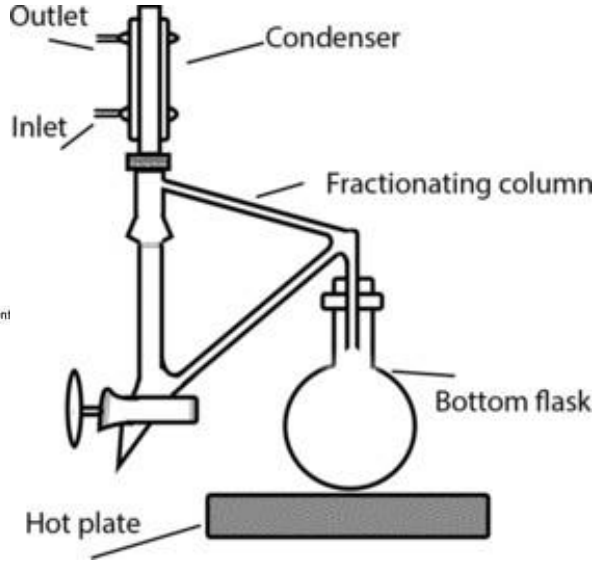

Fig. 5. Clevenger apparatus

Table 4. Extraction of essential oil from oregano applying hydrodistillation

Plan material	Extraction method	Conditions	Yield	Ref.
Oregano	Hydrodistillation	2 h	0.45% w/w	[70]
Oregano	Hydrodistillation	4 h	4.6%, (v/d.w.)	[71]
Oregano	Hydrodistillation	3 h	2.9%	[72]
Oregano	Hydrodistillation	6 h	0.1-0.7% v/w	[73]
Oregano	Hydrodistillation	4 h	1.34%	[74]

In recent years, there is a growing interest in application of supercritical and subcritical extraction with carbon dioxide as a solvent for essential oil isolation from various aromatic plants, including oregano. CO_2 is food-grade and so-called green (non-toxic, non-explosive) solvent, which can be easily removed from the extracted products, yielding high purity extracts [4,76]. The advantages and disadvantages of this high-pressure extraction technique will be further discussed in Chapter 1.3.3 of this thesis.

1.3. Bioactive non-volatile constituents of oregano

1.3.1. Non-polar and polar antioxidants

Acording to Halliwell (2007) antioxidants are any substance that delays, prevents or removes oxidative damage to a target molecule [75]. Antioxidants extends food products period of validity and protects them from deterioration, caused by oxidation (for example color, taste and smell changes). Antioxidant compounds are important because they can protect cells against oxidative damage, which causes aging and chronic-degenerative diseases, such as cancer, cardiovascular disease and diabetes. According to the mode of action, antioxidants are divided into primary (slows or stops the initialization step, interrupt the chain reaction) and secondary (multifunction antioxidants capable of operating free radical reactions stages). The free radical is any atom or molecule species with an unpaired electron. Free radicals are characterized by high chemical reactivity (they are likely to join or give away electrons). Antioxidants give their electrons to free radicals and neutralize them. Many of the food constituent components, such as lipids, proteins, pigments, aromatic compounds are susceptible to oxidation due to the exposure to light, oxygen or metal ions. In order to prevent and/or control oxidative deterioration processes, food products are produced by adding additional natural and/or synthetic antioxidants [76,77]. The main in food industry used synthetic antioxidants are butylated hydroxitoluene (BHT) butylated hydroxyanisole (BHA), propyl gallate (PG), tertbutil hydroquinone (TBHQ). These antioxidants could be used in chewing gum, fats and oils, chips, soups, sauces, potato and confectionery products, oil, nuts, margarine, milk powder. According to Europe Union regulation 1333/2008 (on food additives) synthetic antioxidants can be used alone and/or in combinations with others, but the total amount should not exceed 200 mg/l or 200 mg/kg [78]. However, over the last decade there are some published evidences that synthetic antioxidants have negative effects on human health (circulatory

disorders, high cholesterol, carcinogenic effects), so these food additives are less desirable in food. Therefore, consumer preference and demands for utilization of safer natural plant antioxidants with additional functional (health-beneficial) properties, like vitamin E, glutathione, ascorbic acid, β-carotene, selenium and various phenolic compounds, are constantly increasing [79].

In oregano plant were identified non-polar antioxidants such as apigenine (flavone), eriodictyol (flavanone), dihydroquercetin (dihydroflavonol), dihydrokaemferol (dihydroflavonol). These compounds have such beneficial properties like radical scavenging, antioxidant, anti-inflammatory, anti-allergic, anticancer, antiatherosclerotic, antiaggregational and due of these activities they might be useful for prevention and treatment of human health [80]. Also, carvacrol and thymol as non-polar constituents identified in oregano plants with high essential oil amounts. These compounds stands for remarkable antioxidant properties [80].

Generally, ascorbic acid and phenolic compounds are the major semi-polar and polar antioxidants, present in various foods of plant origin. Phenolic compounds have an aromatic ring with one or more hydroxyl substituents and varies from simple phenolic molecules to highly polymerized compound, whicha are usually referred to as polyphenols. Phenolic compounds by molecular structure are divides into 5 main classes (phenolic acids, flavonoids, stilbenes, coumarins and tannins), among which. phenolic acids and flavonoids are the most widely investigated ones (**Fig. 6**).

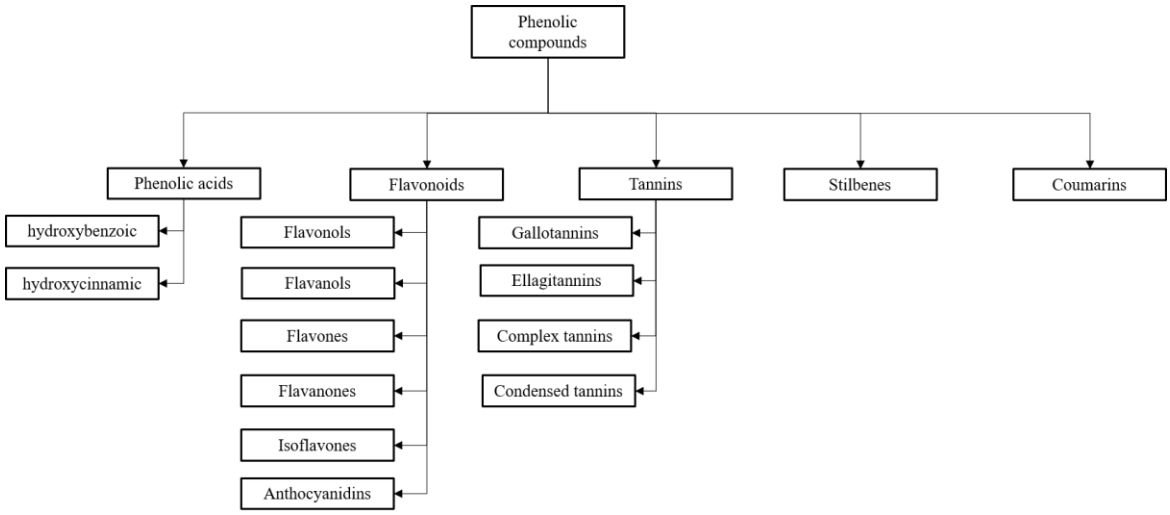

Fig. 6 Phenolic compounds classification

Phenolic acids are synthesized from the shikimate pathway from L-phenylalanine or L-tyrosine [82]. Phenylalanine and tyrosine are very important amino acids in this pathway since these amino acids are the common precursors for the majority of the natural phenolic products (**Fig. 7)**.

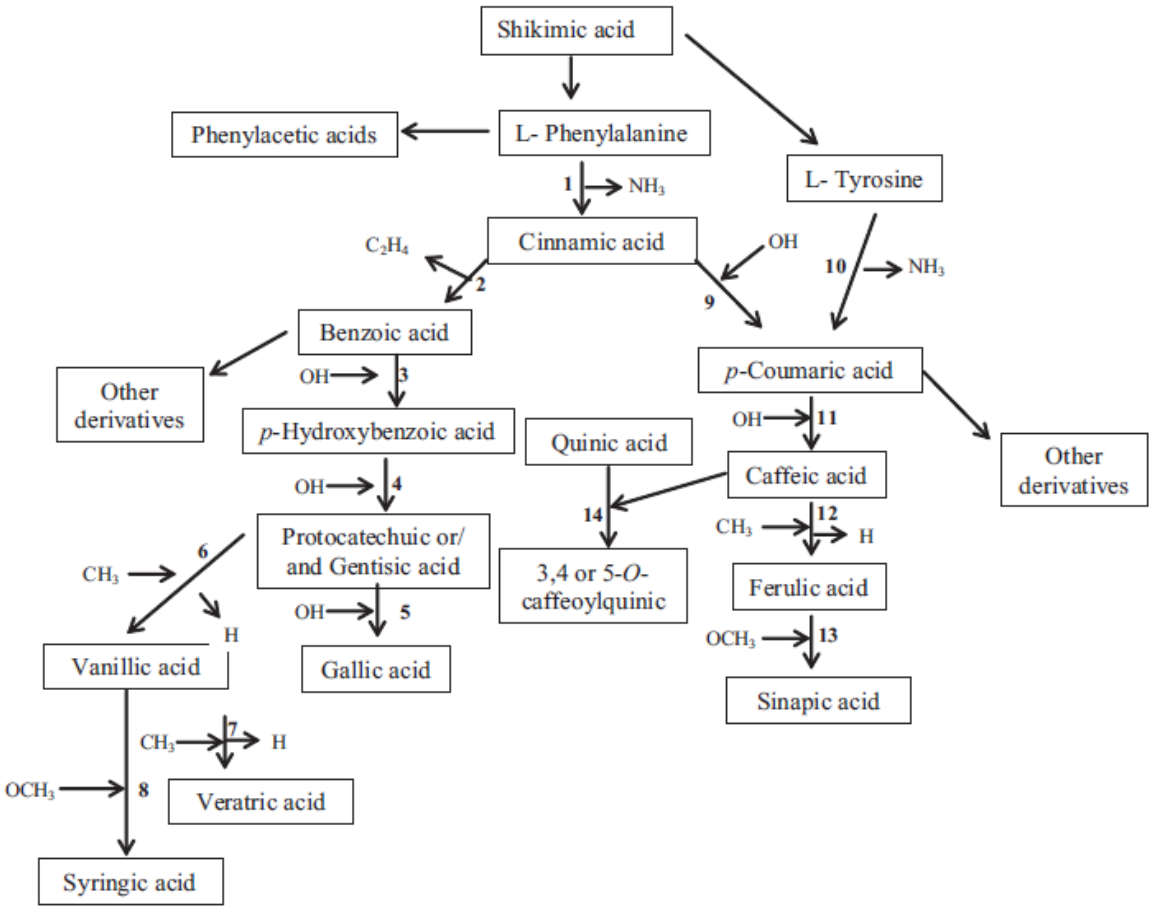

Fig. 7 Phenolic acids biosynthesis (adapted from Heleno et al. [83])

Phenolic acids are divided into two subclasses: hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids. Hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C_6–C_3), with caffeic, ferulic, p-coumaric and sinapic acids being the most common to occurre in plants. After ingestion and absorption, phenolic acids are conjugated by methylation, sulfation and glucuronidation reactions that are controlled by specific enzymes that catalyse these steps [83]. Flavonoids are the largest group of phenolic compounds, they include more than half of all known polyphenols. They are low molecular weight compounds consisting of 15 carbon atoms, arranged in a C_6–C_3–C_6 configuration. The structure consists two aromatics rings A and B, joined by a 3-carbon bridge, which together with an oxygen atom form a heterocyclic ring C (**Fig 8**, apigenin, luteolin, naringenin etc.) [84]. Flavonoids by various C ring modifications are divided into 6 subclasses: flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins and isoflavones. The most common and with large diversity of structures are flavonols and flavones. Differences among the same subclass of compounds are determined by various rings A and B hydroxyl group substituents, which may be methylated, acylated, sulphated, glycosylated. Most of phenolic compounds are present as conjugates with mono- or polysaccharides (mainly, glucose, galactose,

xylose, arabinose), linked to one or more of the phenolic groups and may occur as esters or methyl esters derivatives [85].

Among the different varieties of oregano (wild, grown in Norway; wild, grown in Germany; Greek oregano, grown in Germany, all harvested in 2000) high levels of antioxidants (138.5 mmol/100g, 150.2 mmol/100g, 113.5 mmol/100g, respectively) have been found [86]. The antioxidant potential of oregano extracts has been determined by their ability to inhibit lipid peroxidation, protecting DNA from hydroxyl radical damage, hydrogen peroxide entrapment methods and rancidity testing. In these tests, oregano extracts have shown to be effective, in some cases at levels higher than those exhibited by propyl gallate, BHT and BHA [87]. Also, several important phenolic compounds with potential antioxidant properties *in vitro* and *in vivo* were identified, namely caffeic acid, caffeic acid dimmer – rosmarinic acid, apigenin, luteolin, eriodictyol, naringenin (**Fig. 8**). Rosmarinic acid was the main identified compound (in Greek oregano diethyl ether-soluble extracts the amount was 7.10 mg/100g, in methanol-soluble extracts – 69.39 mg/100g, in commercial oregano methanol-soluble extracts obtained 52.15 mg/100g) [88]. Other authors reported that 649 mg/100 g DW of caffeic acid and 96 mg/100 g DW of neochlorogenic acid could be extracted from oregano [0]. The antioxidant activity depends on the isolated antioxidants polarity and amounts [89]. It is reported that the antioxidant activity of various phenolic compounds depends on their molecular structure, and it is referred as structure-activity relationships. Phenolic acids antioxidant activity depends on functional hydroxy (-OH) groups amount and position comparing to the carboxy (-COOH) groups.

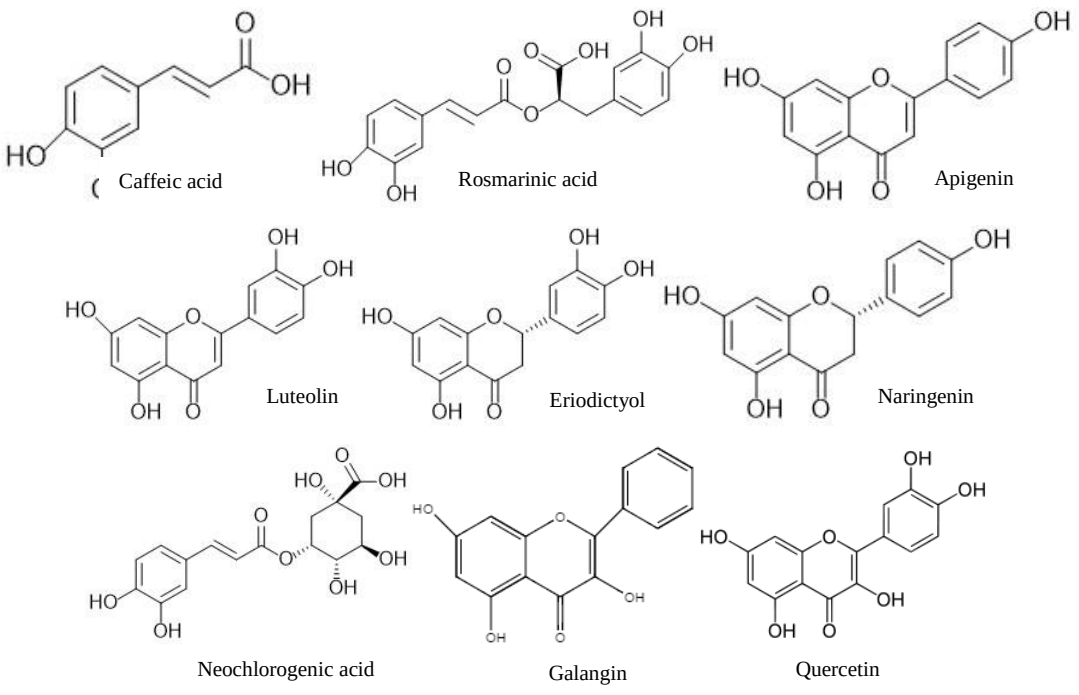

Fig. 8 Selected bioactive phenolic compounds, identified in oregano

Monohydroxy benzoic acids with one -OH group in ortho or para position does not have free radical scavenging activity, opposite to the metha position. The antioxidant activity of phenolic acids increases with increasing degree of hydroxylation. Flavonoids molecular structure-activity relationship much more complicated than phenolic acids. Strong antioxidant activity has flavonoids, in which molecules are these structure features 1) two functional -OH groups in B ring ortho position; 2) C_2-C_3 double bond is conjugated with functional oxo group in C ring fourth position; 3) functional -OH groups in C ring three– and A ring fifth positions [90,91].In addition to antioxidant potential, aqueous extracts of *O. vulgare* and *O. majorama*, as well as flavonoids galangin and quercetin, obtained from methanolic extracts of leaves of oregano (*O. vulgare*), exert important antimutagenic activity against substances commonly found in foods [17]. However, it is also known that flavonoids are a group of phytochemicals that possess hormonal activity. The ability to protect against osteoporosis and cardiovascular diseases, actions attributed to endogenous estrogens such as 17β-estradiol, has supported the estrogenic action of flavonoids. Some of them have antiestrogenic activity because they have been shown to prevent the formation of breast tumors. It has been found that some foods, herbs and spices contain a large amount of substances with estrogenic activity. It was showed that oregano (*O. vulgare*) is one of six spices with the highest ability to bind progesterone, along with verbena, turmeric, thyme, red clover and damiana [17]. Flavonoids have ability to induce human protective enzyme systems. The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases. They also regulate growth factors in plants such as auxin [92,93,94].

1.3.2. Other bioactive constituents

In oregano plant could be found polyunsaturated fatty acids. There are two main families of them – n-3 and n-6. Linoleic acid (n-6) and α-linolenic (n-3) are two main acids which cannot be synthesized by humans. The predominant sources of n-3 fatty acids are vegetable oils and fish. Vegetable oils are the major sources of α-linolenic, also could be found in spinach, and in seeds of flax, linseed, walnuts and others. Fish is the main source of eicosapentaenoic acid and of docosahexaenoic acid. Vegetables also are the main sources of n-6 fatty acids. The most important linoleic fatty acid is found in corn oil, safflower oil, sunflower oil, and soybean oil [95].

1.3.3. Isolation of non-volatile bioactive constituents by conventional extraction techniques

Originally Soxhlet extraction was designed to isolate lipids, but now it is widely used to separate bioactive compounds from various matrixes. In this method, the sample is dried, ground into small particles and transferred to a porous cellulose thimble, which is. placed in the solvent-containing distillation flask. After reaching to an overflow level, the solution of the thimble-holder

is aspirated by a siphon, which unloads the solution, containing extracted solutes, back into the bulk liquid in distillation flask. Disadvantages of this procedure are the following: poor extraction of polar lipids, long extraction time, large volumes of solvents, hazards of boiling solvents, the need to remove solvent from extract, residues of solvent in extract. Solvents for Soxhlet extraction are selected taking into account the properties of target fractions and/or constituents, which are aimed to be retrieved from plant material [96]. For example. hexane is one of the most common non-polar solvent (boiling point – 50-70°C) used to retrieve oily and fatty materials, mixtures of chloroform and methanol at various ratios are employed to extract chlorophyll, acetone and methanol or ethanol are suitable for removal of all polar compounds, while ethyl acetate mostly used for target phenolic compounds extraction [97,98]. Soxhlet extraction with petroleum ether has been used to isolate antioxidants, but now petroleum ether as solvent is used very rare due to its properties (low flash points, extremely volatile, and present significant fire hazard).

Another conventional extraction technique is solid-liquid extraction (maceration or soaking), which is widely used to extract bioactive compounds from various plant materials and also could be applied to retrieve essential oils, for example from citrus fruits. Similarly to Soxhlet extraction, the choice of solvent largely depends on the extraction purpose. Mostly maceration is performed in room temperature, thus allowing to avoid the degradation of heat-labile compounds. Samples could be left soaking for several days (one of the major disadvantages – long extraction time) to obtain the maximum yield. Extraction occurs mainly by diffusive effect, so to speed up the process mechanical shaking and higher temperatures can be applied. After extraction, solid sample particles are separated by filtration [67,68,99].

As given in **Table 5**, Soxhlet extraction to obtain oregano extracts is not very effective, because takes a lot of time and solvent amounts. Depending on conditions, solvents, extraction yield varies from ~3g/100 g DW to 27 g/100g DW. As could be seen in Soxhlet extraction with ethanol, the longer the time – the higher the yield, but in extraction with hexane it is vice versa. In microwave-assisted extraction could be seen that higher temperature increases constituents recovery. Comparing these two extractions with enzyme-assisted extraction could be seen that enzyme-assisted extraction gives way much lower yields. Preferable extraction would be Soxhlet extraction between these three extractions, because highest yields could be obtained (of processes differences). Soxhlet extraction and solid-liquid extraction could take the same time and solvent amount. The efficiency of conventional extraction could be improved, for example, by applying microwaves, ultrasound and/or introducing various enzymes to facilitate extraction process.

Table 5. Extraction of non-polar and polar fractions from oregano and other *Lamiaceae* family plants, applying conventional extraction techniques, microwave and enzyme-assisted extractions

Plan material	Extraction method	Conditions	Yield	Ref.
Oregano	Soxhlet	Ethanol, 4 h	19.25% w/w	[70]
	Soxhlet	Ethanol, 6 h	26.49% w/w	[70]
	Soxhlet	Hexane, 4 h	8.67% w/w	[70]
	Soxhlet	Hexane, 6 h	7.17% w/w	[70]
Oregano	Soxhlet	Hexane, 12 h	3.62%	[100]
Oregano	Soxhlet	Petroleum ether	10.0% w/w	[101]
		diethyl ether	2.9%	
		Ethanol	11.3%	
Sage	Maceration	Hexane 2 h, after – methanol 10 h.	13,88% w/w	[102]
Rosemary			8.7% w/w	
Oregano	Microwave assisted extraction (MAE)	40°C	15.29%	[103]
		70°C	16.60%	
		100°C	22.48%	
		(for all wattage 200, time – 5 min)		
Oregano		petroleum ether	10.0% w/w	[101]
		diethyl ether	2.9%	
		ethanol	11.3%	
Rosemary	Enzyme-assisted extraction (EAE)	Cellulase	1.3%	[104]
		Hemicellulase	1.8%	
		Cellulase/hemicelullase	1.5%	
		10 mg enzymes	1.2%	
		1h, 40°C, followed by hydrodistillation 2 h)		

The examples of the updated conventional extraction techniques could be the following: automated Soxhlet extraction, high-pressure Soxhlet extraction (high pressure is achieved by placing the extractor in a cylindrical stainless-steel autoclave), ultrasound-assisted Soxhlet extraction (the approach uses the conventional Soxhlet glassware, but has the Soxhlet chamber accommodated in a thermostatic bath through which ultrasound is applied), microwave assisted Soxhlet extraction [68,105,106]. Microwave assisted extraction (MAE) is a process of using microwave energy to heat solvents in contact with a sample to partition analytes from the sample matrix into the solvent [107]. Heating with microwaves occurs in the center of sample, so extraction is very fast. The effect of microwave energy is strongly dependent on the nature of both the solvent and the solid matrix. Solvents varies of polarities, from heptane to water. The chosen solvent possesses a high dielectric constant and strongly absorbs microwave energy, the extracting selectivity and the ability to interact with microwaves can be replacable by using mixtures of solvents At times, sample itself interacts with microwaves while the surrounding solvent possesses a low dielectric constant and remains cold [108,109,110]. Ultrasound-assisted extraction (UAE) involves the use of ultrasound ranging from 20 kHz to 2000 kHz [68]. The mechanic effect of

acoustic cavitation from the ultrasound increases the surface contact between solvents and samples and permeability of cell walls. Physical and chemical properties of the materials subjected to ultrasound are altered and disrupt the plant cell wall; facilitating release of compounds and enhancing mass transport of the solvents into the plant cells [111, 112]. Enzymes are ideal catalysts to assist in the extraction, modification or synthesis of complex bioactive compounds of natural origin. Enzyme-assisted extraction (EAE) is based on the inherent ability of enzymes to catalyze reactions with exquisite specificity, regioselectivity and an ability to function under mild processing conditions in aqueous solutions [113]. Enzymes have the ability to degrade or disrupt cell walls and membranes, thus enabling better release and more efficient extraction of bioactives [114,115,116]. The application of these methods for isolation of target fractions from oregano is also exemplified in **Table 5**.

1.3.3. Isolation of non-volatile constituents by high pressure extraction techniques

Supercritical fluid extraction (SFE) is the process of separating constituents from matrix using supercritical fluids as the solvent. Supercritical state is a distinctive state and can only be attained if a substance is subjected to temperature and pressure beyond its critical point. Critical point is defined as the characteristic temperature (T) and pressure (P) above which distinctive gas and liquid phases do not exist. In supercritical state, supercritical fluid cannot be liquefied by modifying temperature and pressure. Supercritical fluid possesses gas-like properties of diffusion, viscosity, and surface tension, and liquid-like density and solvation power [117,118,119]. As depicted in **Fig. 9**, basic SFE system consists of the following parts: CO_2 tank, CO_2 pump, co-solvent vessel and pump, an oven with contains extraction vessel, a heater, a controller to maintain the high pressure inside the system and a trapping vessel. Usually different type of meters, like flow meter, dry/wet gas meter could be attached to the system. The major variables influencing the extraction efficiency are temperature, pressure, particle size and moisture content of feed material, time of extraction, flow rate of solvent, cell shape [117].

A wide variety of solvents is available for use in SFE, including carbon dioxide, nitrous oxide, ethane, propane, n-pentane, ammonia, and water. Currently, carbon dioxide (CO_2) is the solvent of choice for various constituent extraction. CO_2 is nontoxic, nonflammable, noncorrosive, chemically very inert, relatively cheap and yields high purity extracts. The critical temperature of CO_2 (31.1°C) is close to room temperature, and the low critical pressure (74.8 bars) offers the possibility to operate extraction process at moderate pressures, generally between 100 and 450 bar [120]. It is nonpolar, which makes it ideal for extraction of lipids, and non-polar substances. However, the use of carbon dioxide is restricted by its inadequate solvating power for highly polar

analytes and for most pharmaceuticals and drug samples, which can, to some extent, be improved by using an appropriate modifier, e.g. EtOH, acetone, etc. [99].

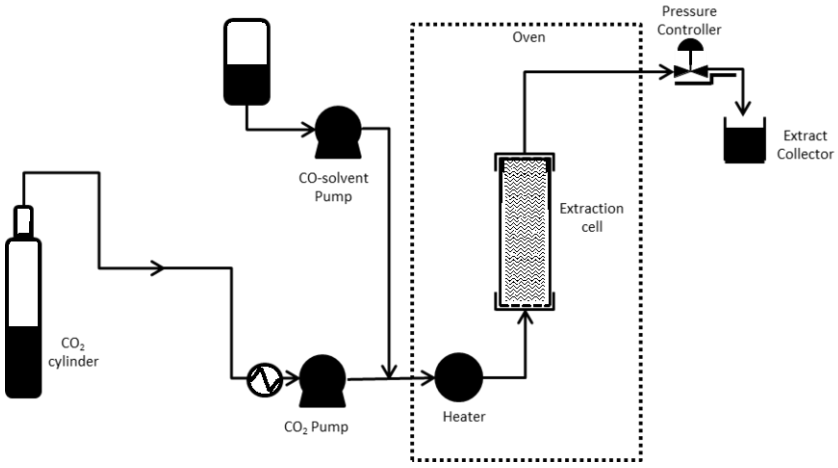

Fig. 9 Supercritical fluid extraction scheme

Another technique of interest in this case is pressurized liquid extraction (PLE): fully automated rapid extraction technique for organic compounds from solid and semisolid matrices [121]. PLE working mechanism is based on moving the solvent through an extraction cell with the sample (**Fig. 10** PLE schem). The cell is heated by direct contact with the oven. The extraction is performed by direct sample contact with the hot solvent in both static and dynamic states. When the extraction is complete, compressed nitrogen eliminates all solvent from the cell to the vial for analysis [122].

Typically, PLE technique uses elevated temperature to increase the extraction efficiency of analytes of interest from the matrix. Elevated pressure is used to keep the solvents in a liquid state as the temperature is increased above their boiling points. By using increased temperatures and pressure solubility of analytes is increased, viscosity of the solvent is reduced and analytes diffusion to the solvent is improved also increased temperature can easily disrupt the strong solute–matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attractions and remove the solute from matrix with ease [123]. Operating at elevated pressure also helps the overall extraction process to take place more rapidly. Pumping solvent through the sample in the cell is easier at elevated pressure. The pressurized solvent is forced into the pores of the sample matrix, resulting more close contact with the analytes in those areas [122].

PLE could be used to determine contaminants in food such as pesticides residues, lipids after acid hydrolysis, additive contents, flavor profiles [68]. PLE is the most suitable for solid and semisolid samples because it provides advantages such as lower solvent volumes, an automatic procedure for the simultaneous extraction for multiple samples, short samples preparing time,

higher yield recoveries, short times for extractions [122]. Typically, extraction is completed in 15–25 min, while consuming only 15–45 mL of solvent.

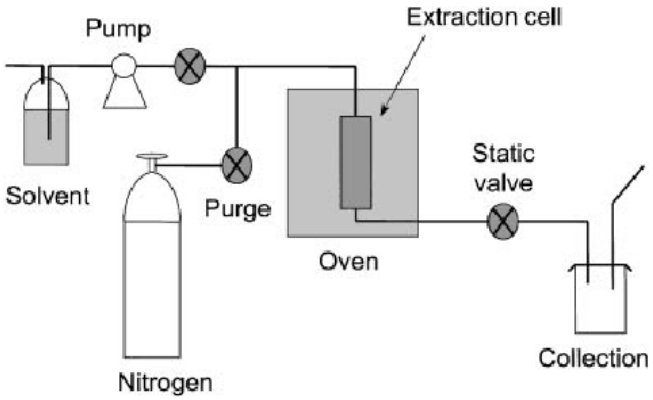

Fig 10 Pressurized liquid extraction (PLE) scheme

From oregano plant isolated constituents yields are presented in **Table 6.** Ac could be seen, using SFE yields are much lower than using PLE extraction. Also, at higher pressure and higher temperature in SFE, higher amount of extract could be isolated. Using PLE recovery increases remarkably. For example, at 200°C using water as solvent yield of 60 g/100 g DW could be reached.

Table 6. Extract yields obtained from oregano by high pressure extraction techniques

Plan material	Extraction method	Conditions	Yield	Ref.
Oregano	Supercritical fluid extraction (SFE)	10 MPa and 40°C	1.00%, w/w	[125]
		30 Mpa 100°C	1.5%	[74]
		30 MPa and 40°C	1.59-3.18%	[126]
Oregano	Pressurized liquid extraction (PLE)	50°C, EtOH	~5%	[124]
		100°C, EtOH	~13%	
		150°C EtOH	~19%	
		200°C, EtOH	~38%	
		50°C, H_2O	~23%	
		100°C, H_2O	~32%	
		150°C, H_2O	~51%	
		200°C, H_2O	~60%	
		25:75 H_2O/EtOH	~26%	
		50:50 H_2O/EtOH	~32%	
		75:25,H_2O/EtOH (temp. 100°C)	~34%	

1.4. Domestic and industrial applications of oregano and its products

As discussed in Chapters 1.1-1.3 of this thesis, *O. vulgare* is classified as a medicinal and spice plant with a wide range of health promoting properties: antiseptic, antioxidant, antispasmodic, carminative, antiallergic, antidiabetic, antimutagenic, anticarcinogenic, antimicrobial, anti-inflammatory features [32]. Medicinal, culinary, cosmetics uses and positive effect of oregano plant and various oregano products (extracts, essential oils and hydrolats) are

summarized in the **Table 7.** Most the essential oils are classified as GRAS (generally recognized as safe) substances, while their use in food as preservatives is often limited due to flavor considerations [46].

Table 7. Application areas of oregano and its products

Application area	Oregano products	Functions
Food industry	Fresh and/or dried oregano, spice mixes	Flavouring substances to sauces, salads, drinks. Functional food ingredients
Cosmetics industry	Oregano (Origanum vulgare) essential oil, tincture, organic oregano hydrolats and hydrosols	Antibacterial, tonic, stimulates microcirculation refreshing moisturizes the skin. These properties help against acne, cellulite, hair growth. Cures dandruff, stimulates hair growth, nail fungus, skin disorders and rashes
Pharmaceutical industry	Fresh or dried oregano, tea from oregano leaves, oregano oil (especially with high carvacrol amount), oregano oil capsules	Immuno stimulatory effects, stimulates digestion, antiparasitic effects (internally). Helpful against muscle aches, colds, mouth infections, treatment of wounds. Good against allergies, capable of killing a variety of fungi, antioxidant, prevents aging, arthritis, rheumatism, bacterial infections, diarrhea, immune system boost, migraine, muscle pain, asthma, psoriasis, pain killer, sinus

Both freshy harvested and dried oregano herb is commonly added to foods at both household and industrial levels (salad, sauces, soups, meat and other products) to maintain its flavor and increase food oxidative stability. Similarly, essential oils and extracts (obtained with non-toxic solvents) could be added to oils in order to control lipid oxidation process. However, industrial applications of antioxidant-enriched extracts (other than essential oils) might be limited due to the undesirable flavor of foods, which additionally indicates the need to study the changes of the bioactive substances during deodorization processes.

There are different ways how oregano-based cosmetic and pharmaceutical products are marketed: capsules of essential oils (or their components), pure essential oils, diluted essential oils, hydrolats (pure or mixed with another hydrolats or essential oils), oregano-based syrups, etc.

However, due to the high thymol and carvacrol levels, oregano essential oil should not be utilized pure for medicinal and cosmetic purposes (appropriate dilutions are required). Some people may experience stomach problems, when ingesting oregano oil (or even the herb itself). Those who are allergic to plants from the *Lamiaceae* family (mint, lavender, sage, and basil) should also avoid this oil, as they may also develop an allergic reaction. Also, oregano oil is also not advisable for infants and children. Pregnant or nursing women are also discouraged from using oregano oil both topically and orally, as it can encourage blood circulation within the uterus, which

deteriorates the lining that encompasses the fetus within the womb. Oregano oil also has a potential to induce menstruation, and may be dangerous to unborn child.

In addition to the above discussed application areas, plant essential oils represent an alternative for the protection of crops against pests. Some essential oils and their components possess a broad spectrum of activity against insects, mites, fungi and nematodes, such as *Rhyzopertha dominica, Tribolium castaneum,* and *Sitophilus oryzae,* pest attacking grains [17].

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$, Sigma-Aldrich, Steinheim, Germany), 2,2-diphenyl-1-picrylhydrazyl hydrate ($DPPH^{\bullet}$, free radical, 95%), 3,4,5-trihydroxybenzoic acid (gallic acid, 99%, Sigma-Aldrich, Steinheim, Germany), 2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoic acid (Fluorescein (FL), Fluka Analytical, Bornem, Belgium), 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%, Sigma-Aldrich, Steinheim, Germany), Folin & Ciocalteu's phenol reagent ((2M), Fluka Analytical, Bornem, Belgium), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (Lach-Ner, Brno, Czech Republic), Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), Na_2CO_3 (Sigma-Aldrich), H_2SO_4, NaOH, H_3PO_4, (Sigma-Aldrich), HCl (35-38%, Chempur, Piekary Slaskie, Poland), squalene (99%, Supelco Analytical, Bellefonte, PA, USA), acetonitrile, methanol, dichlormetane, pentane, hexane (HPLC grade, Sigma-Aldrich Chemie, Steinheim, Germany), boron trifluoride (24% methanol solution, Acros organics, Geel, Belgium), a mixture of C_8-C_{32n}-alkanes (Sigma Chemical Co., St. Louis, MO), microcrystalline cellulose (20 μm, Sigma-Aldrich, St. Louis, MO, USA), catalytic tablet (K_2SO_4, $CuSO_4$, Sigma-Aldrich), ASE filters (Glass Fiber_(X)_Cellulose, Dionex Corporation, Sunnyvale, CA,USA), diatomaceous earth (100 % SiO_2, Dionex Corporation, Sunnyvale, CA, USA), cotton-wool (Bella-cotton, Poland), glycine (>99%, Sigma-Aldrich, Bornem, Belgium), D-(+)-glucose (>99%, anhydrous, Acros Organics, Geel, Belgium), acetone, methanol (analytical grade, Sigma-Aldrich, Poole, UK) ethanol (96.3%, food grade, Stumbras, Kaunas, Lithuania), hexane (PENTA Chemikalien, Mainaschaff, Germany), nitrogen liquid (AGA SIA, Riga, Latvia), carbon dioxide gases, nitrogen gases (99.9%, Gaschema, Jonava region, Lithuania).

2.2. Preparation of plant material

Oregano (*Origanum vulgare L.*) was harvested in 2015 summer, plant was grown in Babtai, Lithuania. Stems and leafs were dried and prior to analysis were kept in a dark, well-ventilating room at ambient (20°C) temperature. Oregano was ground to different particle size fractions (1

mm, 0.5 mm, 0.2 mm) with ultra-centrifugal rotor mill (Retsch ZM200, Retsch GmbH, Germany) (8000 rpm). Ground material was kept in tightly closed, dry glass jars, in dark, well-ventilated place prior to the analysis.

2.3. Determination of the selected chemical composition indices of oregano

2.3.1 Moisture content

To the heated, dry, constant weight glass with cap and rod, three (2.7230, 2.7215, 2.7219 ±0.002 g) samples g of oregano (particle size 0.2 mm) was weighted. Sample was stirred and dried in the oven at 100-105°C. After 3 hours, samples were cooled for 25 minutes in desiccators and the weight was measured. The weighting procedure was repeated every hour until constant weight (variation between two results is 0.001-0.005 g). Experiments were performed in triplicate. Moisture content was calculated using the formula below (g/100g).

$$x = \frac{(m_1 - m_2)*100}{m_1 - m}; g/100g \quad (1)$$

m – glass with cap and rod weight g; m_1 – glass weight with sample before drying g; m_2 – glass weight with sample after drying, g.

2.3.2. Mineral content

To dry, constant weight crucibles, three (2.0237, 2.0243, 2.0218 ±0.002 g) samples of oregano (0.2 mm fraction) was added. Crucibles with samples were putted on electric hotplate and heated until smoke has stopped to form. After that, crucibles were transferred to muffle (internal temperature of 600-650°C) and kept for ~16 hours. Samples were burned until two consecutive weight measures differ 0.0001 to 0.0005 g. Experiments were performed in triplicate. Ash (mineral) content, expressed as a percentage, is calculated by the following formula:

$$x = \frac{(m_2 - m)*100}{m_1 - m}; g/100g \quad (2)$$

m – crucible weight, g; m_1 – crucible weight with sample, g; m_2 – crucible weight with burned sample, g.

2.3.3. Protein content by Kjeldahl method

Tree (1.0021, 1.0020, 1.0010 ±0.002 g) samples of oregano (0.2 mm fraction) were weighted to special Kjeldahl flask, filled with 20 ml conc. H_2SO_4 and catalyst tablet (3.5 g K_2SO_4, 0.4 g $CuSO_4$). Samples were mineralized until solution in the flask became transparent (heating intensity 60%, time – 90 min). The solution was distillated with automatic steam distillation system under the following conditions: 3 s NaOH and 3 s H_3BO_4 filing parameters, distillation time 300 min, steam intensity 80%. Distillate was collected in flask, followed with the addition of Tashiro indicator and titration with 0.01 N HCl until the colour change from light green to grey-violet. Control sample (20 ml conc. H_2SO_4) was prepared and analysed following the above described

conditions. Experiments were performed in triplicate. The nitrogen content, expressed as a percentage, was calculated using the following formula:

$$x = \frac{0.0014 * A}{m} * 100; g/100g \quad (3)$$

A – 0.1N HCl amount, used for distillate titration, ml; m – sample weight, g; 0.0014 – nitrogen amount equivalent 1 ml 0.1 N HCl. Protein material amount is calculated by multiplying the amount of nitrogen from the conversion factor 6.25.

2.4. Isolation of bioactive oregano constituents by conventional extraction techniques

2.4.1. Hydrodistillation

Hydrodistillation for essential oils isolation of dried oregano was carried out in a Clevenger-type apparatus during 4 hours from 300 g (applicable for 1 mm, 0.5 mm and 0.2 mm fractions were used 300 g of dried herb, in case of unground material – 100 g of dried herb. Each sample was mixed with ~1,8 l distilled H_2O. Essential oils amount was measured volumetrically, putted to bottles and kept in a freezer prior to further analysis of volatile compound composition was investigated by gas chromatography-mass spectroscopy (GC-MS). Solid residues and aqueous phase were kept in a freezer and then, before further researches were lyophilized.

2.4.2. Soxhlet extraction

Soxhlet extraction was performed in an automated Soxhlet extractor EZ100H (Behr Labor-Technik, Düsseldorf, Germany) as a standard technique (AOAC reference method [127]), using 20±0.001 g of ground oregano (1 mm, 0.5 mm or 0.2 mm fraction) or 10±0.001 g of unground sample, loaded into a filter paper, rolled up tightly and inserted into an inner tube of the apparatus. Non-polar fractions were isolated using hexane, while residues (0.2 mm fraction) were further extracted with acetone. The rate of extraction was 1 cycle per 5 min, total extraction time – 360 min (6 hours).

Organic solvents were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland). Extract yields were determined gravimetrically (±0.001 g) and expressed as g/100 g DW. Additionally, after hexane extraction, the packages were dried, weighted with 0.0001 g accuracy and the lipid content, expressed as a percentage, was calculated by the following formula:

$$x = \frac{(a-b)*100}{m}; g/100g \quad (4)$$

a – sample weight with filter paper before extraction, g; b – sample weight with filter paper after extraction, g; m – sample weight taken for extraction, g.

Dry extracts were kept under the nitrogen flow for 15 min to remove organic solvent residues and stored at -18°C prior to the analysis. The solid residue was collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis. Experiments were performed in triplicate.

2.4.3. Solid-liquid extraction (SLE)

SLE was performed in a thermostatically controlled shaker from 20±0.1 g of oregano (0.2 mm fraction) and 150 mL of hexane or acetone (*solid: liquid* ratio *1:7.5*) at the following conditions: temperature 50°C (for hexane extraction), 20 and 40 °C (for acetone extraction), time 360 min, 800 rpm, followed by the rapid cooling and centrifugation (9000 rpm, 10 min) and filtration. Organic solvents from the optically clear supernatants were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland). Dry extracts were kept under the nitrogen flow for 15 min to remove organic solvent residues and stored at -18°C prior to the analysis. SLE-He and SLE-Ac extracts yield was determined gravimetrically (±0.001 g) and expressed as g/100 g DW. The solid residues were collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis.

2.5. Isolation of bioactive oregano constituents by high-pressure extraction techniques

2.5.1. Supercritical CO_2 extraction (SFE-CO_2)

SFE-CO_2 was performed in a supercritical fluid extractor Helix extraction system (Applied Separation, Allentown, PA, USA) by modified procedure of Kraujalis and Venskutonis (2013) [13]. Each extraction was carried out using 16±0.001 g of ground plant material (oregano and solid residues after hydrodistillation), which was placed in a 50 cm^3 cylindrical extractor of 14 mm inner diameter and 320 mm length. The absorbent cotton wool was placed on the top and in the bottom of the extraction vessel to avoid particle release to the system. The volume of CO_2 consumed was measured by a ball float rotameter and a digital mass flow meter in standard liters per minute (SL/min at standard state (P_{CO2} = 100 kPa, T_{CO2} = 20°C, ρ_{CO2} = 0.0018 g/ml). The static extraction time was 10 min for all experiments. Extraction conditions were set as follows (**Table 8**): extraction pressure 275 and 450 bar, temperature 25, 40, 50 and 70ºC, time 90, 210 and 300 min, modifier 5% EtOH. After extractions, the yield of extracts was determined gravimetrically (±0.001 g) and expressed as g/100g DW. Extracts were collected to an opaque bottle and kept in a freezer until further analysis. Solid residues were kept dry, well-ventilated place prior to the further analysis. All extractions were performed in triplicates.

Table 8. SFE-CO_2 extraction condition

Sample	Particle size, mm	Temperature, °C	Pressure, MPa	Time, min	EtOH, %
Oregano	0.2	40	27.5	300	-
Oregano	0.2	40	45	300	-
Oregano	0.2	25	45	210	-
Oregano	0.2	50	45	210	-
Oregano	0.2	70	45	210	-
Oregano	0.2	40	45	210	5
Oregano	0.2	50	45	210	5
Oregano	0.2	70	45	210	5
Oregano (after hydrodistillation)	0.2	70	45	210	-

2.5.2. Pressurised liquid extraction (PLE)

2.5.2.1. Optimization of extraction parameters

PLE was performed in ASE-350 (Thermo Scientific Dionex, Sunnyvale, CA, USA) apparatus as by modified procedure of Kraujalis et al. (2013) [14]. To prepare samples for extraction, 7 g of plant material was mixed with 7 g diatomaceous earth (1/1, w/w) and placed to 66 ml stainless-steel extraction cells, with two cellulose filters in the both ends to avoid particle release to the system. Initially, in order to optimize PLE parameters, oregano residues after choosen SFE-CO_2 (50°C, 450 bar, 210 min) were extracted with acetone under different conditions: temperature 40-120°C, time 15-60 min (**Table 9**). Afterwards, multistep extractions were conducted with ethanol, H_2O and ethanol/ H_2O mixture (1:1, v/v), utilizing solid plant material residues after optimized PLE with acetone under the following conditions: temperature 40 and 120°C, time 30 and 60 min (**Table 10**). Additionally, in order to compare conventional and high-pressure extraction efficiencies, oregano plant material was extracted with hexane under the following conditions: extraction temperature 50°C, time 5min x 3 cycles. The system pressure (103 bar or 10.3 MPa), pre-heating time (5 min), cell flush volume (100%) and purge time (120 s) with nitrogen to collect the extracts in the vials was kept constant for all PLE experiments. Organic solvents were evaporated with rotary evaporator at different pressure (acetone, hexane – 180 bar, ethanol – 80 bar) at 45°C by Buchi Rotavapor R-210 (BUCHI Labotechnic, Switzerland).

Table 9. Levels of independent variables for PLE-Acetone parameter optimization (1st step PLE)

Experimental factors	Variable levels		
	-1	*0*	*+1*
Extraction temperature (T, ºC)	40	80	120
Extraction time (τ, min)	15 (5 min x 3 cycles)	37.5 (12.5 min x 3 cycles)	60 min (20 min x 3 cycles)

Table 10. 2nd and 3rd step PLE parameter optimization

1st step PLE			2nd step PLE			3rd step PLE		
Solvent	Temperature, °C	Time, min	Solvent	Temperature, °C	Time, min	Solvent	Temperature, °C	Time, min
Acetone	120	30 →	Ethanol	40	30			
Acetone	120	30 →	Ethanol	120	60			
Acetone	120	30 →	Ethanol	120	30			
Acetone	120	30 →	Ethanol/ H_2O	120	30			
Acetone	120	30 →	Ethanol	120	30 →	H_2O	120	30
Acetone	120	30 →	Ethanol/ H_2O	120	30 →	H_2O	120	30

EtOH/H_2O, H_2O extracts were additionally freeze-dried (-50ºC, 0.5 mbar) to remove residual water. The yields of extracts were determined gravimetrically (±0.001 g) and expressed as g/100g DW, extract were kept in brown glass bottles in the freezer prior to the analysis. Solid residues were kept dry, well-ventilated place prior to the further analysis. All extractions were performed in triplicates

2.5.2. Preparation of reference compounds and determination of browning development

For the UV-visible absorbance measurements, PLE extracts were dissolved in methanol to a final concentration of 1000 μg/mL. Absorbances at 420 nm were recorded with with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY).

In order to evaluate browning development during PLE, standard COST glycine-glucose melanoidins were prepared under water-free reaction conditions, following the procedure of Kitrytė et al. (2012) [128]. In a 500 ml glass beaker, 50 mmol of glycine (3.754 ± 0.001 g) and 50 mmol of D-(+)-glucose (9.008 ± 0.001 g) were well-mixed, placed in an oven (Memmert, equipped with a fan), and heated without cover at 125 °C for 2 h. After heating, the flask with brown to black coloured reaction products was cooled down to room temperature in a desiccator (up to 2 h, until the weight of the reaction products remained stable) and grinded to a fine powder. Standard glycine-glucose melanoidin solutions (1500μL) at various concentrations (2.5-30mg/mL) were used for calibration. The browning development of PLE extracts was expressed as standard glycine-glucose melaniodins equivalents (g StandMel/g extract and per g StandMel/g DW) by means of dose-response curves for glycine-glucose melanoidins: y=0.0327x+0.0165, R^2=0.9995

2.6. Antioxidant activity assessment of oregano extracts and solid residues

For the *in vitro* antioxidant activity measurements in Folin-Ciocalteu's, $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC assays, various extracts after different steps of extraction were dissolved in acetone-methanol mixture (1:9, v/v) and further diluted with methanol to a final concentration from 25μg/mL to 4000 μg/mL. Water-soluble fractions after hydrodistillation were dissolved in dist. H_2O to a final concentration from 1 μg/mL to 200 μg/mL.

Antioxidant capacity of starting plant material and solid residues after various steps of extraction was evaluated by QUENCHER method (Gökmen et al., 2009 [129]). As previously described by Kitrytė et al. (2014), stock mixtures were prepared with 0.2 mm fractions and microcrystalline cellulose at a concentration of 500 μg/mg. Final solid dillutions for analysis were prepared at concentrations of 0.8 μg/mg to 190 μg/mg.

2.6.1 Total phenolic content (TPC) by Folin-Ciocalteu's assay

Folin-Ciocalteu's assay was carried out by the procedure of Singleton, Orthofer and Lamueal-Raventós (1999) [130], with some modifications. For the analysis, 150μL of sample (200-2000μg/mL) or MeOH (blank) were mixed with 750μL Folin-Ciocalteu's reagent (2M), previously diluted with distilled water (1:9, v/v), and after 3 min of reaction, 600μL of Na_2CO_3 solution (75g/L), left in dark for 2 hours and absorbance was measured at 760 nm with with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY).

For the QUENCHER procedure, as previously described by Kitrytė et al. (2014) [131], 10 mg of sample (2-190μg/mg) or cellulose (blank) were mixed with 150μL of distilled water, 750μL Folin-Ciocalteu's, 600μL of Na_2CO_3 solution, vortexed for 15 s, shaken at 250 rpm in the dark for 2 hours, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 760 nm with spectrometer. Gallic acid solutions (150μL) at various concentrations (0-80μg/mL) were used for calibration. The TPC of extracts and solid samples was expressed as gallic acid equivalents (mg GAE/g sample and mg GAE/ g DW) by means of dose-response curves for gallic acid. Extracts calibration curve: $y = 0.0117x + 0.0081, R^2 = 0.9976$, QUENCHER: $y = 0.0108x + 0.0024, R^2 = 0.9999$.

2.6.2. The DPPH• scavenging assay

The DPPH• assay was carried out by the method of Brand-Williams, Cuvelier and Berset (1995) [131], with some modifications as follows. To a 1000 μL of a ~89.7 μmol/L (final absorption adjusted to 0.800±0.010 AU at 517 nm) DPPH• methanolic solution 500 μL of sample (10-1000μg/mg) or MeOH (blank) were added. All mixtures were left in dark absorbance was measured after 2 hours at 517 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY).

For the QUENCHER procedure, 10 mg of sample (0.8-12μg/mg) or cellulose (blank) were transferred to a centrifugation tube, mixed with 500 μL of MeOH and 1000 μL of a ~89.7 μmol/L DPPH• methanolic solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 517 nm. Trolox solutions (500 μL) at various concentrations (0-50 μmol/L MeOH) were used for calibration. $TEAC_{DPPH}$ of extracts and solid samples (before and after extraction) were calculated

by means of dose-response curves for Trolox. Extract: $y = 1.3284x + 1.8618, R^2 = 0.9974$, QUENCHER: $y = 1.422x + 1.3594, R^2 = 0.9966$

2.6.3. The ABTS$^{\bullet+}$ scavenging assay

The ABTS$^{\bullet+}$ assay was carried out by the method of Re at al. (1999) [133] with slight modifications. Firstly, phosphate buffered saline (PBS) solution (75 mmol/L; pH 7.4) was preperad by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of distilled water. The ABTS$^{\bullet+}$ solution was prepared by mixing 50 mL of ABTS$^{\bullet+}$ (2 mmol/L PBS) with 200 µL $K_2S_2O_8$ (70 mmol/L) and allowing the mixture to stand in the dark at room temperature for 15-16 h before use. The working solution was prepared by diluting the ABTS$^{\bullet+}$ solution with PBS to obtain the absorbance of AU 0.700±0.010 at 734 nm. To a 1500 µL of working ABTS$^{\bullet+}$ radical solution 25 µL of sample (200-4000µg/mg) or MeOH (blank) were added, mixtures left in dark for 2 hours and absorbance was measured at 734 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY).

For the QUENCHER procedure, 10 mg of sample (0.8-12µg/mg) or cellulose (blank) were mixed with 25 µL of MeOH and 1500 µL of working ABTS$^{\bullet+}$ radical solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 734 nm. Trolox solutions (25 µL) at various concentrations (0-1500 µmol/L MeOH) were used for calibration. $TEAC_{ABTS}$ of extracts and solid samples were calculated by means of dose-response curves for Trolox. Extract: $y = 0.0659x + 0.2274, R^2 = 0.9989$, QUENCHER: $y = 0.0686x + 1.4856, R^2 = 0.9962$

2.6.4. Oxygen radical absorbance capacity (ORAC) assay

ORAC of the samples was evaluated as described by Prior et al. (2003) [134] by using fluorescein as a fluorescent probe. In the 96-well black opaque microplates, 25 µL sample (25-1000µg/mg) or MeOH (blank) was mixed with 150 µL of fluorescein solution (14 µmol/L) preincubated for 15 min at 37°C, followed by a rapid addition of 25 µL of AAPH solution (240 mmol/L). The microplate was immediately placed in the FLUOstar Omega reader (BMG Labtech, Offenburg, Germany), automatically shaken prior to each reading and the fluorescence was recorded every cycle (1min x 1.1), total 120 cycles. The 485-P excitation and 520-P emission filters were used. Raw data were exported from the Mars software to Excel 2003 (Microsoft, Roselle, IL) for further calculations. Antioxidant curves (fluorescence versus time) were first normalized and from the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as $AUC = 1 + \sum_{i=1}^{i=150} \frac{f_i}{f_0}$, where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

For the QUENCHER procedure, 10 mg of sample (0.9-48μg/mg) or cellulose (blank) were mixed with 150 μL PBS solution (75 mmol/L) and 900 μL of fluorescein solution (14 μmol/L PBS), vortexed for 15 s, shaken at 250 rpm for 60 min in the dark and centrifuged (450 rpm, 5 min). Optically clear supernatant (175 μL) was transferred to the 96-well black opaque microplates, preincubated for 15 min, at 37°C, followed by a rapid addition of 25 μL of AAPH solution (240 mmol/L) as a peroxyl radical generator using a multichannel pipette. The fluorescence was recorded every cycle (1 min x 1.1), total 150 cycles. Further experimental and data handling were the same as reported for extract analysis. Trolox solutions (150 μL) at various concentrations (0-500 μmol/L PBS) were used for calibration. $TEAC_{ORAC}$ of extracts and solid samples were calculated by means of dose-response curves for Trolox. Extract: $y = 0.1297x + 3.5824, R^2 = 0.9576$, QUENCHER: $y = 0.2062x + 1.2901, R^2 = 0.9846$.

2.6.5. Measurement of oxidation induction period by Oxipres

The effect of oregano extracts on the oxidative stability of commercial rapeseed oil was tested by instrumental Oxipres method (Trojáková et al., 1999 [135]; Laubli and Bruttel, 1986 [136]). The samples were prepared by mixing rapeseed oil with 1%, 2.5%, 5% SFE-CO_2, 1% acetone and 1% ethanol PLE extracts (as control sample was used pure rapeseed oil). Five grams of prepared (or control) sample were placed in a reactor tube and thermostated at 110°C under oxygen atmosphere at 5 bar in Oxipres apparatus (Mikrolab, Aarhus, Denmark), which measures pressure changes due to the absorption of oxygen consumed for oil oxidation. The induction period (IP) was calculated as the time after which the pressure began to decrease abruptly (its end was measured from the cross-section point oftangents ofthe first part and the subsequent part ofthe curve recording the pressure changes) [135]. Each measurement was done in duplicate.

2.7. Phytohemical characterization of oregano extracts

2.7.1 Volatile compound analysis by gas chromatography-mass spectroscopy (GC-MS)

Essential oils after hydrodistillation, SFE-CO_2 and Soxhlet extracts were analyzed by GC-MS analysis, as previously described by Baranauskienė and Venskutonis (2013) [137]. GC–MS analyses were performed using a Perkin Elmer Clarus500 GC coupled to a Perkin Elmer Clarus 500 series mass selective detector (Perkin Elmer Instruments, Shelton, USA) in the electron impact ionization mode at 70 eV, the mass range was m/z 29–550. Volatile compounds were separated using an Elite-5 MS capillary column (dimethylpolysiloxane, 5% diphenyl), 30 m length, 0.25 mmi.d., 0.25 μm film thickness (Perkin Elmer Instruments, Shelton,USA). The oven temperature was programmed from 50°C (2 min) to 280°C (10 min) at the rate of 5°C min^{-1}. Carrier gas helium was adjusted to a linear velocity of 36.2 cm s^{-1} at 50°C or 1.0 mL min^{-1} volumetric flow. Split mode was used at ratio of 1:20 and an injector temperature of 250°C.

The identity of the components was assigned by comparing their Kováts retention indices (KI) relative to C_8–C_{32n}-alkanes (SigmaChemical Co., St. Louis, MO), obtained on nonpolar DB-5 column with those provided in literature (Adams, 2009), and by comparing their mass spectra and retention time with those of reference substances and by comparison with the Wiley (6^{th}) and the NIST Mass Spectral Library (Version 1.6 d, 1998) and particular literature data if available. Kováts retention indice values were calculated using an n-alkane ladder as follows:

$$I = 100 * (n + \frac{t_r - t_{r\,(n)}}{t_{r\,(N)} - t_{r(n)}}); \quad (5)$$

I - Kovats retention index; n-the number of carbon atoms in the smaller n-alkane; N-the number of carbon atoms in the larger n-alkane, t_r – retention time.

2.7.2. Non-volatile compound analysis by UPLC/ESI-QTOF-MS

Phytochemical composition of aqueous extracts after hydrodistillation (ungrounded, 0.2, 0.5, 1 mm fractions), Soxhlet extracts (ungrounded, 0.2, 0.5, 1 mm fractions), SFE-CO_2 extract at optimal conditions, SLE extracts (mechanical shaking acetone and hexane extracts)PLE extracts (PLE-Acetone, acetone, hexane, ethanol, ethanol/water, water extracts, were screened on an Acquity UPLC system (Waters, Milford, USA) equipped with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germant), binary solvent delivery system, an auto sampler with a 10 μL sample loop, column manager, photodiode array (PDA) detector and an Acquity BEH C18 column (1.7 m. 50 x 2.1 mm, i.d.), as previously described by Kraujalyte and Venskutonis (2013) [138] with following modifications. The mobile phase initially consisted of eluent A (0.4 v/v formic acid in ultra-pure water), followed by an increase from 0% to 100% of eluent B (acetonitrile) 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 and the equilibration time of 1 min. Separation of compounds was performed at 25°C; the column was equilibrated for 1 min before each run; the flow rate 0.4 mL/min; extract concentration 1 mg/ml; injection volume 1 μL. The effluent (monitored at 254 nm) from the PDA detector was introduced directly into the UHR-TOF mass spectrometer equipped with an ESI source. MS data was recorded in ESI negative ionization mode. The capillary voltage was maintained at +4000 V with the end plate offset at -500 V. Nitrogen was used as the drying and nebulizing gas at a flow rate of 10.0 L/min and a pressure of 2.0 bar. For the instrument control and data acquisition, the Compass 1.3 (HYStar 3.2 SR2) software was used. Preliminary peak identification was carried out by comparing accurate masses of compounds with hose reported in literature sources and free chemical databases (Metlin, Chempspider).

2.7.3. Squalene determination by high performance liquid chromatography (HPLC)

An HPLC was used for the quantitative determination of squalene in 25-70°C, Et40-Et70°C, 70°C HD SFE-CO_2, SLE-He, SLE-Ac, Soxhlet-He, Soxhlet-Ac, PLE-He, PLE-Ac, PLE-EtOH extracts, according to Gruszka and Kruk [139] with slight modifications. Perkin Elmer Series 200HPLC system was equipped with C_{30} reverse-phase column (particle size 5 µm, 250 mm × 4.6 mm) applying isocratic elution with acetonitrile:methanol:dichlormethane (72/22/6, v/v/v). Injection volume was 20 µl and flow rate 1 ml/min. Squalene was detected by UV detector at 214 nm wavelength, its elution time was ~3 min. For samples were diluted to a final concentration of 0.1%. Squalene was identified by comparing the retention time of peaks to pure standard solutions, which were prepared at concentrations of 0–1 mg/ml using mobile phase. HPLC conditions were set the same as described before. The calibration curves (peak area versus injected amount) were used to determine the quantity of squalene in the samples. Analyses were performed in triplicate.

2.7.4. Fatty acid composition analysis by gas chromatography (GC-FID)

Fatty acid composition analysis was performed by the procedure of Moreda et al. (2001) [140]. For triglycerides esterification and free acids saponification, 0.5 g extract (50°C SFE-CO_2, Soxhlet-He, SLE-Ac, PLE-He, PLE-Ac) and 4 ml of methanolic NaOH (0.5 N) was poured into 50 ml round-bottomed flask and heated with condenser until disappearance of the fatty phase (5-10 min). After esterification, over the top of condenser 5 ml of 24% boron trifluoride/methanol complex was poured and boiled for 2 min., then cooled to room temperature. The sample was diluted with 5 ml n-hexane and the same amount of NaCl was added, well-shaken and left still until layers separated. The top hexane phase was collected with a Pasteur pipette and stored at 4ºC until analysis. For analysis, 100 µl of hexane phase was diluted with 900 µl pure GC-grade hexane. Analysis was carried out with gas chromatograph HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) using a flame ionization detector with a pole SPTM-2560 column (100 m long, 0,25 mm internal diameter the adsorbent layer of 0,20 µm (Supelco, Bellefonte, PA, USA). Oven temperature was programmed from 80°C to 240°C and increasing every 4°C/min. Injector temperature – 220°C and detector – 240°C. Injected amount of sample – 1µl. For compounds identification, a mixture of 37 fatty acids (SupelcoTM). were used as standards. Fatty acid methyl esters were identified by the retention time and the percentage of fatty acid composition was calculated comparing peak areas to the corresponding reference compounds.

2.8. Experimental design

Response surface methodology (RSM) using central composite design (CCD) was employed to determine the influence of two independent variables (temperature and time) on the PLE extraction yield, total phenolic content and browning development and to identify optimum

conditions for polar constituent isolation from oregano. Extraction time (t=15-60 min) and temperature (T=40-120°C) were selected as independent variables with three levels for each of them. The complete design consisted of 13 experimental runs with 5 central points. Center points conditions were these: time – 37.5 min, temperature - 80°C. The software Design-Expert trial version 8.0.6.1 (Stat-Ease Inc., Minneapolis, MN, USA) was used for data analysis and model building, as previously reported by Kraujalis et al. (2013) [13]. The number of experiments was defined by the formula:

$$N = (2f + 2f + c); \quad (6)$$

where f – the number of factors; c – the number of center points.

2.9. Statistical analysis

Mean values and standard deviations were calculated using MS Excel 2016. One-way analysis of the variance (ANOVA), followed by the Tukey's posthoc test to compare the means that showed significant variation ($p < 0.05$), also bivariate correlation analysis and Pearson correlation coefficients between different antioxidant activity indices were performed and calculated using GraphPad Prism 6.01 software (2012).

3. RESULTS AND DISUSSION

3.1. Chemical composition of oregano and aroma compounds obtained by hydrodistillation

For the purposes of this research, oregano, grown in Lithuania, was fractionated into essential oil, non-polar and polar constituent fractions applying conventional (hydrodistillation, solid-liquid extraction (SLE) and Soxhlet extraction with different solvents) and high pressure (SFE-CO_2 with and without EtOH modifier and PLE with different polarity solvents under the various extraction conditions) extraction techniques. In order to characterize plant material and determine extraction efficiencies, qualitative composition of essential oils yield was determined, broad screening of antioxidant activity of different extracts and plant material prior and after extractions were carried out, as well as oil stability with different kind of extracts was assessed, and phytochemical composition of the selected non-volatile fractions, including fatty acid composition and squalene content determination, was analysed.

First of all, oregano plant material chemical composition was identified. Moisture, lipid, protein, mineral content is presented in **Table 11**. Comparing analyzed values with data from USDA Food Composition Databases [23], it could be seen that investigated protein content (10.1 g/100g) is very similar with given in database (11 g/100g). Lipid content varies, but in database its suggested value is 10.3 g/100g, in this research this value is lower – 7.6 g/100g. Determined mineral content was 7.13 g/100g, specified mineral composition was not identified. According to European Spice Association (ESA), mineral content in dried oregano could be 10

g/100g [141] and moisture content 12g/100g, while in this study obtained moisture value was 5.47 g/100g. Blagojevic et al. (2013) indicated mineral content and moisture in dried oregano samples. Mineral content average in their samples was 6.75 g/100g, moisture content average – 10.65 g/100g. Mineral content was similar in this research like in these authors work, moisture content was almost 2-fold lower [142]. Chemical composition mostly depends on oregano growing conditions, climate, minerals in soil and what kind of anatomical parts were used, because in different anatomical parts could be accumulated different amounts of these components. Moisture content depends in what kind of conditions oregano were kept and what kind of drying method was used.

Table 11. Chemical composition of oregano plant material, amounts expressed as g/100g DW

Index	**Amount, g/100g**	**Index**	**Amount, g/100g**
Moisture content	5.47±0.33	Protein content	10.06±0.32
Lipid content	7.61±076	Mineral content	7.13±0.18

Essential oil (EO) is a concentrated hydrophobic liquid fraction, containing different volatile aroma compounds, which has a very strong scent, extracted from plants. Essential oils could be extracted by different extractions methods, but the most common is traditional hydrodistillation. In this study was carried out hydrodistillation with four different particle size fractions (unground, 1 mm, 0.5 mm, 0.2 mm), all at the same conditions (4 hours). Obtained essential oil yields are presented in **Fig 11**. The highest yield of essential oil was obtained from unground fraction (0.21 ml/100 g DW) and reducing particle size essential oil amount decreased as follows:1 mm (1.2-fold) > 0.5 mm (1.2-fold) > 0.2 mm (1.2-fold). According to the widely accepted taxonomic reference for genus six variable *O. vulgare* subspecies were discerned. *O. vulgare L*. subsp. *glandulosum* (Desfontaines) Ietswaart, *O. vulgare* L. subsp. *hirtum* (Link) Ietswaart and *O. vulgare* L. subsp. *gracile* (Koch) Ietswaart are the subspecies rich in volatiles (southernmost area), while those in Central and Northern Europe (*O. vulgare* L. subsp. *virens* (Hoffmannsegg et Link) Ietswaart, *O. vulgare* L. subsp. *vulgare* L. and *O. vulgare* L. subsp. *viride* (Boissier) Hayek are considered to be poor sources of essential oils [143]. Plants with high essential oil amount (2% or more) usually collect large amounts of phenolic monoterpenes deriving from 'cymyl'-pathway (mainly carvacrol and/or thymol and their precursos γ-terpinene and p-cymene) [144]. Such plant material has wide commercial potential because of these monoterpenes oregano flavor [145]. In individuals with intermediate or poor oil content the 'cymyl'-pathway seems to be less active or inactive at all.

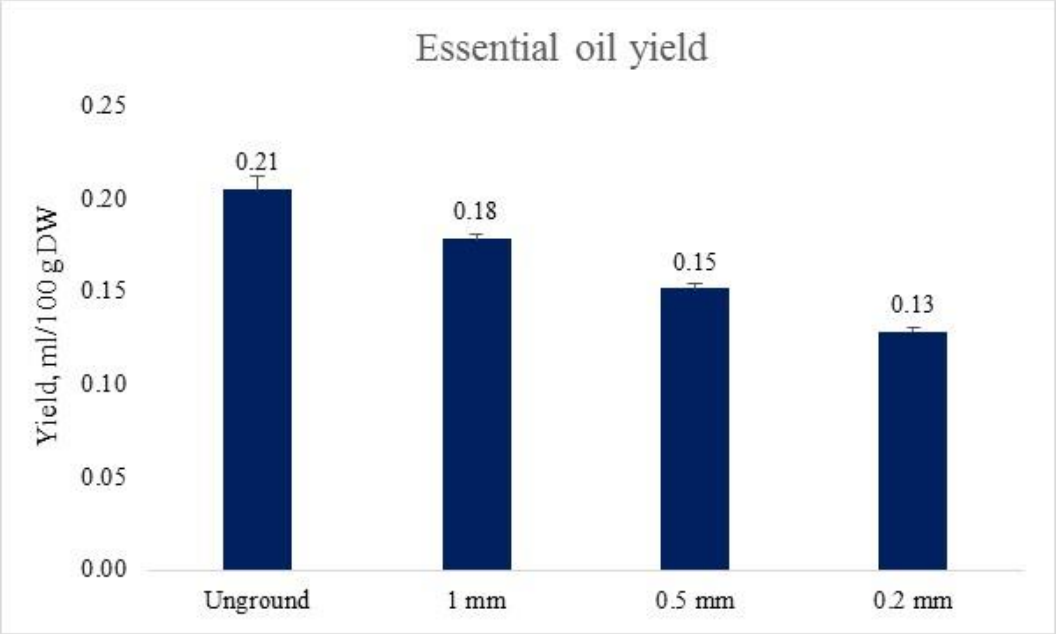

Fig. 11 Isolated essential oil yields

The monoterpene fraction from these plants comprises higher amount of acyclic compounds (linalool, linalyl acetate, β-ocimene, mircene) and/or bicyclic 'sabinyl'-compounds (sabinene and *E/Z*-sabinene hydrate and their acetates) and/or bornane type compounds (e.g. camphor, borneol, bornyl acetate) and is often accompanied by high amounts of sesquiterpenes (such as e.g. β-caryophyllene, germacrene D, bicyclogermacrene, α-muurolene, β-caryophyllene oxide) [145,146,147]. Qualitative volatile compound composition in essential oils from oregano samples of various particle sizes is reported in **Table 12**. Quantities of individual volatile compounds were expressed as percentage of the total GC peak area (semi-quantitative analysis) and the identification was based on comparison of: (1) their mass spectra with Wiley (6^{th}) and the NIST Mass Spectral Library and (2) calculated Kovats retention indices with those reported in literature. In addition, aroma properties descriptors and odor threshold values, when available, were reported for individual volatiles too (**Table 12**). On the basis of the main essential oil constituents of oregano, growing in Lithuania, it was assigned to sabinene/germacrene D chemotype.

As given in **Table 12**, 25 volatile constituents were identified in essential oil from oregano, obtained after 4 hours of hydrodistillation. The main identified compounds were sabinene (amount varies from 8.14% to 33.18% in different particle size fractions), germacrene D (6.79%-12.33%), α-caryophyllene (5.90%-11.62%), *E*-α-ocimene (3.19%-9.57%) and α-phellandrene (2.48%-4.66%) (**Fig. 12**). In different fractions, main compounds amount quite differs. Sabinene amount in unground fraction (33.18 %) 4-fold higher than in 0.2 mm fraction (8.14 %). In 0.5 mm and 1 mm fractions sabinene amount is respectively 1.7-fold 1.4-fold lower (20.84 % and 22.41 %). The highest germacrene D amount (12.33%) was obtained in 0.2 mm fraction, and this is 1.8-fold higher than in 1 mm fraction (6.79%-the smallest quantity) in 0.5 mm fraction and unground

fraction germacrene D amount were quite similar (7.51 % and 7.76 % respectively). The highest α-caryophyllene amount was in 0.2 mm fraction (11.62%) and this is almost 2-fold higher amount than in unground fraction (5.90%). In 0.5 mm and 1 mm fraction this compound amount was similar – 6.91% and 6.44 %. Highest amount of E-α-ocimene was obtained in 1 mm fraction – 9.67%, 3-fold higher than in 0.2 mm fraction (3.19%). In 0.5 mm fraction E-α-ocimene amount was 2.4-fold higher than in 0.2 mm fraction (7.60%). In unground fraction this compound amount was in between 1 mm and 0.2 mm fraction – 8.99 %. Highest α-phellandrene amount was obtained in 1 mm fraction (4.66%), almost 2-fold higher than in 0.2 mm fraction (2.48%). In 0.5 mm fraction α-phellandrene amount was almost high as in 1 mm fraction (4.57%). In unground fraction obtained amount was 3.31%. Sabinene gives oregano wood, citrus, pine, spice odors, *E*-α-ocimene gives fruit, floral, wet, cloth odor, α-phellandrene – citrus, mint, pepper, α-caryophyllene adds sweet, clove, dry odors. The classes of identified compounds include bicyclic monoterpenes (α-pinene, sabinene), alicyclic monoterpenes (α-myrcene, *E*-α-ocimene, *Z*-β-ocimene) monocyclic monoterpenes (limonene, p-cymene, α-phellandrene), monoterpene alcohols (eucalyptol, linalool, terpinen-4-ol, α-terpineol), bicyclic sesquiterpenes (α-caryophyllene, δ-cadinene, caryophyllene oxide) and others. In this study, essential oil yield was higher in ungrounded material, because during the milling process certain EO amount could be lost. Also during hydrodistillation some compounds can be easily extracted from the smaller plant particles, be effected by heat, split up and remain in the aqueous phase. Essential oil chemotypes are defined by its main monoterpene compound but it becomes difficult when considering the whole monoterpene pattern. Due to the lack of commercial interest in oil-poor O. vulgare especially populations from the Northern Mediterranean and of Central and Northern Europe are still poorly explored. When considering that most of the consumed *O. vulgare* plant material is still wild collected limited knowledge is insofar relevant as unknown biodiversity may be irrecoverably lost by overexploitation of populations. Within *O. vulgare* a highly active cymyl-pathway and especially the accumulation of carvacrol and/or thymol seems to be one prerequisite for a high essential oil yield. In Lithuania were presented different oregano plant groups, one rich in 1.8-cineole, other one in β-cymene rich plants [147,149]. Essential oil constituents amounts also can be influenced by the plant material drying method. Figiel et al. (2010) carried out study where was investigated drying methods influence on essential oil composition. Were tested these drying methods: convective drying (CD), vacuum-microwave (VM) and convective pre-drying combined with vacuum-microwave finish drying (CPD–VMFD). In fresh oregano herb main identified compounds were carvacrol (9.09 g/kg DW), thymol (8.44 g/kg DW), γ-terpinene (4.87 g/kg DW), 2-hexen-1-ol (2.37 g/kg DW), p-cymene (2.01 g/kg DW).

Table 12. Essential oils isolated from different particle size fractions (unground, 1 mm, 0.5 mm, 0.2 mm) by hydrodistillation composition and amounts (%), odor threshold and description

Compound	KI on DB-5*	KI on DB-5**	0.2 mm	0.5 mm	1 mm	Unground	Description	Odor threshold	
								detection	recognition
α-Pinene	947	939	0.32±0.01^{a}	0.94±0.01^{b}	1.03±0.02^{c}	1.12±0.03^{d}	cedarwood, pine, resin, sharp,	0.23-105	0.00036-29
1-Hepten-3-ol	963	959	4.44±0.19^{b}	4.15±0.32^{b}	4.10±0.1^{b}	2.35±0.50^{a}	earthy green fruity mushroom	0.07	
Sabinene	981	975	8.14±0.11^{a}	20.84±3.69^{b}	22.41±1.15^{b}	33.18±0.62^{c}	woody,citrus pine spice		1.5-3
α-Myrcene	996	991	0.50±0.02^{a}	1.21±0.06^{b}	1.51±0.03^{d}	1.30±0.03^{c}	balsamic, fruit, herb, must		0.0445-0.15
Limonene	1031	1029	1.01±0.02^{a}	1.69±0.22^{b}	1.83±0.05^{b}	1.07±0.02^{a}	citrus, mint	0.038 ppm	
p-Cymene	1041	1033	2.29±0.08^{a}	3.08±0.06^{d}	2.69±0.06^{c}	2.49±0.10^{b}	fresh citrus woody spice	7.2	0.012-2.4
α-Phellandrene	1044	1032	2.48±0.05^{a}	4.57±0.57^{c}	4.66±0.15^{c}	3.31±0.05^{b}	citrus, mint, pepper, wood		2.9-3.9
E-α-Ocimene	1045	1050	3.19±0.06^{a}	7.60±0.12^{b}	9.57±0.14^{d}	8.99±0.09^{c}	fruity floral wet cloth		
Eucalyptol	1050	1039	1.97±0.03^{a}	2.84±0.17^{c}	3.46±0.05^{d}	2.12±0.04^{b}	camphor, cool, mint, sweet	0.003-2	0.0006-0.36
Z-β-Ocimene	1057	1050	0.56±0.01^{a}	1.67±0.03^{b}	2.28±0.06^{c}	2.63±0.04^{d}	Sweet, herb	34 ppb	
1.3.8-p-Menthatriene	1102	1111	0.72±0.04^{b}	0.99±0.01^{c}	1.17±0.09^{d}	0.59±0.02^{a}			
Linalool	1110	1107	1.41±0.02^{d}	1.13±0.02^{c}	1.03±0.04^{b}	0.66±0.01^{a}	citrus floral green blueberry	0.0005-6	0.0014-0.11
Terpinen-4-ol	1202	1188	2.21±0.04^{b}	3.40±0.41^{c}	3.42±0.22^{c}	1.76±0.03^{a}	pepper woody earth musty		>3
α-Terpineol	1218	1207	1.33±0.02^{d}	1.01±0.02^{c}	0.94±0.03^{b}	0.54±0.01^{a}	pine lilac citrus woody floral	0.01-0.86	0.0125-110
α-Bourbonene	1406	1388	4.41±0.11^{d}	2.97±0.14^{c}	2.62±0.05^{b}	1.92±0.03^{a}			
α-Caryophyllene	1447	1444	11.62±1.13^{b}	6.91±0.54^{a}	6.44±0.72^{a}	5.90±0.67^{a}	sweet woody spice clove dry		1.5-13
Humulene	1464	1455	3.87±0.07^{c}	2.41±0.14^{b}	1.89±0.02^{a}	1.94±0.06^{a}	balsamic, hop, spice	160 ppb	
Aromadendrene	1477	1464	1.41±0.02^{c}	0.60±0.04^{a}	0.69±0.02^{b}	0.60±0.01^{a}	cucumber, floral, vanilla		
Germacrene D	1498	1485	12.33±0.22^{c}	7.51±0.22^{b}	6.79±0.21^{a}	7.76±0.35^{b}	woody spice		
α-Farnesene	1514	1506	1.28±0.03^{a}	1.31±0.09^{a}	1.45±0.05^{b}	1.83±0.11^{c}	citrus herbal lavender bergamot	20 ppb	
α-Muurolene	1523	1500	2.08±0.02^{c}	0.74±0.08^{b}	0.28±0.11^{a}	-	woody		
ç-Elemene	1524	1393	2.19±0.72^{b}	1.62±0.09^{b}	0.77±0.01^{a}	2.15±0.33^{b}	sweet, wood		
δ-Cadinene	1541	1523	2.97±0.05^{c}	1.67±0.15^{b}	1.50±0.03^{a}	1.44±0.03^{a}	thyme herbal woody dry		
Spathulenol	1582	1578	4.35±0.14^{c}	2.38±0.23^{b}	1.94±0.04^{a}	1.72±0.07^{a}	fruit, herb		
Caryophyllene oxide	1596	1583	3.12±0.05^{d}	1.85±0.12^{c}	1.51±0.04^{a}	1.66±0.05^{b}	sweet fresh dry woody spicy	64 ppb	

Referred average values of six determinations ± SD. Different superscript letters within the same line indicate significant differences (one way ANOVA, Tukey's test $p<0.05$); KI* - Kováts retention indices calculated against C8–C32n-alkanes, KI** - Kovats indices reported in literature (Adams, 2009). Odor threshold range and odor description were found in VCF (Volatile Compounds in Food 16.3) database [148]

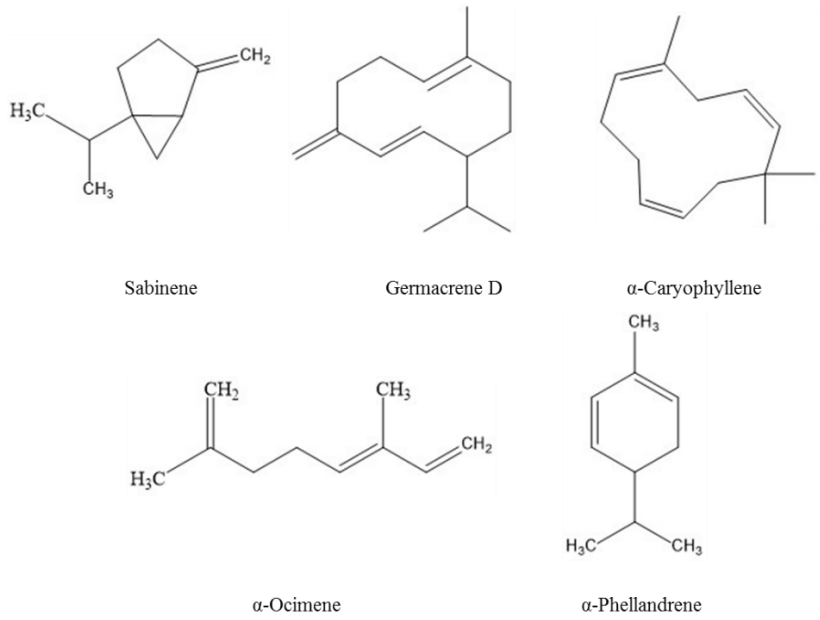

Fig 12. The main compound identified in oregano EO's

After convective drying these compounds amounts dropped as follows: 3.64 g/kg DW, 2.14 g/kg DW, 1.49 g/kg DW, 0.39 g/kg DW, 0.65 g/kg DW, respectively. After vacuum-microwave (VM) drying amounts were these: 6.85 g/kg DW, 5.60 g/kg DW, 5.26 g/kg DW, 0.58 g/kg DW, 2.25 g/kg DW. After convective pre-drying combined with vacuum-microwave finish drying (CPD–VMFD): 4.40 g/kg DW, 2.61 g/kg DW, 1.34 g/kg DW, 0.60 g/kg DW, 1.32 g/kg DW. The drying method had remarkable effects on the of the volatile compounds in by various methods dried oregano. The use of hot air in any part of the drying process of fresh oregano caused important losses of volatile compounds. The dried oregano samples with the highest content of volatile compounds were those obtained by VM without convective pre-drying followed by samples dried CPD–VMFD [150].

Looking at the previous reports of other researchers, Govaris et al. (2010) isolated essential oil from oregano (*O. vulgare subsp. hirtum*), obtained at spring harvest from local cultivation in the central Greece, were hydrodistilled in a Clevenger type distillation apparatus for 2 h. Isolated essential oil constituents yielded 3.6 ml/100 g DW [151]. Essential oil chemical composition also has been analysed and main constituents were: p-cymene (5.18%), γ-terpinene (2.80%), thymol (4.82%), carvacrol (80.15%). Amount expressed as percentage of the total peak area of the chromatograms without correction factors. Esen and coworkers (2007) isolated essential oils from wild-growing *Origanum vulgare subsp. hirtum* samples from the Marmara region of Turkey, cultivated in Yalova with hydrodistillation for 3 hours. The analyses showed that wild and cultivated O. *vulgare subsp. hirtum* oils obtained from wild plants contained carvacrol (7.5-82.9% and 5.3-85.4%, respectively), and thymol (0.3-60.1% and 0.3-68.0%, respectively) as the main components. Other constituents obtained in wild and cultivated samples were: p-cymene (6.4-

31.1% and 2.8-31.6%), γ-terpinene, (0.1-7.8% and 3.0-19.5%), linalool (0.1-0.4% and 0.1-0.3%) [152]. In Pesavento et al. (2015) studies, main identified compounds were carvacrol (71.8%), p-cymene (11.6%), β-caryophyllene (2.7%), linalool (1.8%), γ-terpinene (1.7%), [153]. Essential oils were obtained by steam distillation from *Origanum vulgare*. Also, other compounds such as α-pinene, myrcene, limonene, α-terpineol, humulene, δ-cadinene were obtained in this research as well as published in literature. Asensio et al. (2015) by 2 hydrodistillation obtained essential oils from four different Argentinian oregano types and main compounds identified in them were trans-sabinene hydrate (17.9%-28.12%), thymol (12.09%-18.58%), terpinen-4-ol (6.18%-9.52%), γ-terpinene (7.09%-9.8%), orto-cymene (5.13%-7.78%) [154]. As other authors studies showed almost in all of them main compounds were thymol and carvacrol, that means that for their samples were used from carvacrol/thymol chemotype, probably grown in countries with high air temperature. In this study carvacrol and thymol traces in essential oils were identified, but theirs amount was lower than 0.1 % so their existence in essential oils were not presented, because oregano plant was grown in Lithuania, where high carvacrol and thymol amounts in plant rarely is noticeable. Stefanaki et al. (2016) reported study about isolated essential oils from *Origanum onites L* growing wild in different conditions. For essential oils isolation were used Clevenger-type apparatus, for 2 hours. Studies were focused on essential oil content and 5 main compounds amount – p-cymene, γ-terpinene, borneol, thymol and carvacrol. Obtained essential oils yield varied from 3 to 7 ml/100 g DW. Carvacrol was the main compound obtained in all 42 tested samples from different growing condition (from 69.0 to 92.6%). In this study was pointed out one thing – growing conditions such as geological substrate, altitude, bioclimatic area, geographical location have a small impact on main compound – carvacrol amount [155].

3.2. Isolation of non-polar constituents by conventional and high pressure extractions

In order to isolate non-polar constituents from *O.vulgare* and compare different extraction method (conventional *vs* high pressure) efficiency, plant material was subjected to (**Fig 13**): (1) Soxhlet extraction with hexane; (2) solid-liquid extraction (SLE) with hexane; (3) supercritical carbon dioxide extraction (SFE-CO_2) at different pressure and temperature conditions with or without 5% ethanol modifier; (4) pressurized liquid extraction (PLE) with hexane. Non-polar fraction yields, expressed as g/100 g DW of plant material, under the different extraction conditions are reported **Table 13**.

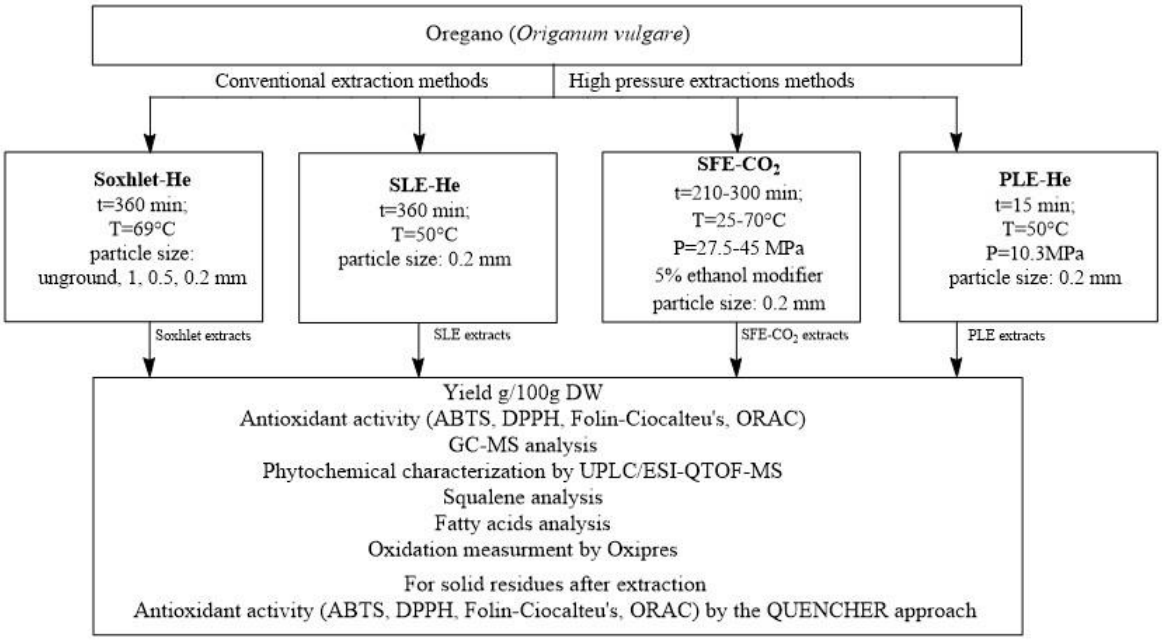

Fig 13. Non-polar constituents isolation scheme

Table 13. Conventional and high pressure extractions parameters and isolated non-polar constituents (Soxhlet, SLE, SFE-CO_2) yields.

Sample		Extraction parameters			Yield
		Pressure, MPa	*Temperature, °C*	*Time, min*	*g/100g DW*
Conventional extraction methods					
Soxhlet-He	unground	-	69	360	2.35±0.11^{d}
Soxhlet-He	1	-	69	360	3.02±0.13^{def}
Soxhlet-He	0.5	-	69	360	3.28±0.12^{f}
Soxhlet-He	0.2	-	69	360	3.21±0.05^{f}
SLE-He	0.2	-	50	360	1.78±0.11^{bc}
High pressure extraction methods					
SFE-CO_2	0.2	45	25	210	1.31±0.12^{a}
SFE-CO_2	0.2	45	40	210	1.51±0.04^{ab}
SFE-CO_2	0.2	45	50	210	2.01±0.05^{c}
SFE-CO_2	0.2	45	70	210	2.30±0.00^{d}
SFE-CO_2(5% EtOH)	0.2	45	40	210	2.92±0.09^{de}
SFE-CO_2 (5% EtOH)	0.2	45	50	210	3.13±0.11^{ef}
SFE-CO_2 (5% EtOH)	0.2	45	70	210	2.76±0.01^{d}
SFE-CO_2 (after HD)	0.2	45	70	210	2.00±0.11^{c}
PLE-He	0.2	10	50	15	3.08± 0.09^{ef}

Referred average values of three determinations ± SD. Different superscript letters indicate significant differences (one way ANOVA, Tukey's test $p<0.05$);

As reported in **Table 13**, Soxhlet extraction was performed utilizing unground plant material and samples of three different particle sizes (0.2, 0.5, 1 mm). Non-polar fraction yield from unground oregano was the lowest (2.35 g/100g DW), while extraction efficiency was significantly improved by 22-28% reducing particle size of plant material up to 0.2-1 mm (3.02-3.28 g/100 g DW). Since the hexane-soluble fraction yield from differently ground samples did not differ significantly, on average 3.17 g/100 g DW of lipophilic constituents could be extracted from oregano (particle size variation 0.2-1 mm) using this conventional extraction technique. Generally, reducing of particle size could facilitate better recovery of target constituents due to the enhanced

disruption of plant cells and higher surface area. However plausible temperature increase should be taken into account during the grinding process, since this could lead to the undesirable loss of heat-labile plant constituents.

Other conventional method used to obtain non-polar constituents was SLE. Oregano sample of the smallest particle size (0.2 mm) amounted only 1.78 g/100g DW of hexane-soluble fraction, which was significantly lower (1.3-1.8-fold) as compared to the Soxhlet yield of the same fraction. These results could be explained by the lower extraction temperature applied in the case of SLE. Previously, Radha Krishnan et al. (2014) run out a Soxhlet extraction on oregano in order to determine the effect of different plant particle sizes on extraction yield. Authors established, that, when particle sizes increase, the extraction yield decreases. Also results of these authors showed that after 6 hours of extraction, 16.91 g/100 g DW of non-polar constituents were removed from oregano (0.4-0.63 mm) using hexane as extraction solvent [158].

As alternative to conventional extraction, two high pressure extraction methods were used in this research to isolate non-polar oregano constituents, namely SFE-CO_2 and PLE with hexane. For SFE-CO_2, there is three main parameters to influence extraction yields – pressure, time and temperature. Usually the temperature for SFE-CO_2 extraction varies from 40°C (to protect thermolabile compounds) to 100°C (for higher molecular weight compounds) [74]. In order to set extraction pressure and time, kinetic experiments were conducted using oregano sample of 0.2 mm particle size at constant extraction temperature of 40°C and pressures of 27.5 and 45 MPa. The yield of SFE-CO_2 extract was measured every 30 minutes for 300 min in total. As it could be seen from **Fig. 14**, constant extraction yield of g/100 g DW (27.5) and of g/100 DW (45 MPa) was reached after 210 min of extraction, therefore this extraction time was further applied for all other SFE-CO_2 experiments.

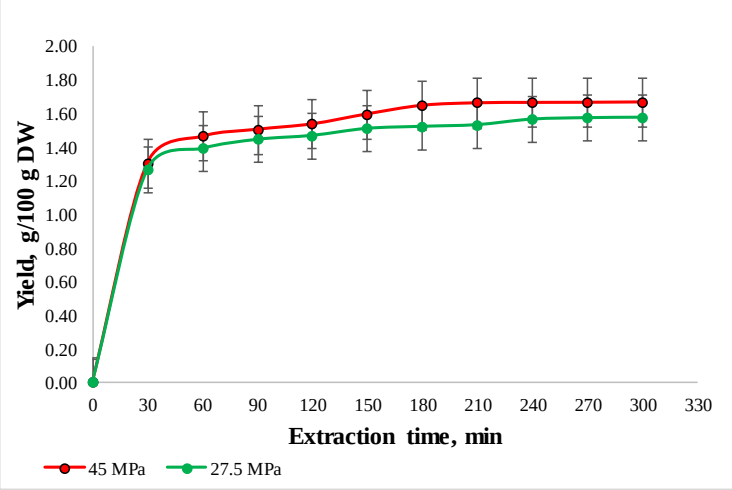

Fig. 14 Kinetics of SFE-CO_2 extraction at 40°C under different extraction pressure

Extraction efficiency was increased by ~8% at elevated extraction pressure, therefore 45 MPa were preferable for SFE-CO_2 of oregano. SFE-CO_2 extraction yields can significantly rely on selected temperature as well. The results of this study are in agreement with our findings, since a noticeable increase in yield was observed elevating extraction temperature from 25°C to 70°C (pressure 45 MPa, time 210 min): 25°C (1.31 g/100 g DW) < 40°C (1.51 g/100 g DW) < 50°C (2.01 g/100 g DW) < 70°C (2.30 g/100g DW). These yields could be additionally improved by 26-58% introducing 5% of EtOH modifier to CO_2 flow. The ability of ethanol modifier to increase the SFE-CO_2 extract yield could be explained by the fact, that the presence of ethanol increases the solubility and extraction efficiency of semi-polar and polar constituents during SFE-CO_2 process. Ethanol also decreases matrix interactions and reduces viscosity, enhancing CO_2 flow. As reported by in research with chamomile, extracted yield at 30 MPa pressure was ~4 g/100 g DW, at 68.9 MPa - ~5g/100g DW. Modifier addition increased extraction yields significantly: 5% of EtOH increased yield at 30 MPa to ~5 g/100g DW, at 68.9 MPa to ~6 g/100g DW; 20% of modifier at 30 MPa isolated ~7.5 g/100 g DW of semi-polar, polar constituents, at 68.9 MPa was obtained ~8.5 g/100 g DW yield [156]. Since CO_2 is considered as non-polar solvent, one sample was applied to SFE-CO_2 after hydrodistillation (while water extracts polar constituents), to obtain how much non-polar constituents could be isolated. Obtained yield was – 2.00 g/100 g DW, which is similar to yield obtained after SLE with hexane and SFE-CO_2 at 50°C.

Stamenic and others (2014) reported varying yields for Greek oregano (*Origanum heracleoticum* or *Origanum vulgare L. ssp. Hirtum*) at different SFE-CO_2 conditions: 0.74 g/100 g (30 MPa, 40°C, CO_2 flow rate 2.7l/min), 1.02 g/100 g (10 MPa, 40°C, CO_2 flow rate 4.5l/min), 1.5 g/100 g (30 MPa, 100°C) [74]. Comparing this study results with those reported by Stamenic, yield recovery was 44-52% higher in our study. It may be concluded, that neither higher flow rate (2.7-4.5 L/min) nor higher extraction temperature does not result in higher yield if SFE-CO_2 is carried out at low pressures. Recently, another study was conducted by Saúl García-Pérez and co-workers (2017). These researchers carried out SFE-CO_2 with varying percentage of EtOH modifier: (1) 16% EtOH at 10 MPa and 40°C; (2) 32% EtOH at 10 MPa and 60°C; (3) 32% EtOH at 30 MPa and 40°C. In that study CO_2 flow rate was kept constant and very high – 13.6 l/min, total extraction time of 60 min (extraction with modifier was conducted for 40 min, while remaining 20 min only with CO_2). These conditions and high modifier amount resulted in relatively high extraction yields: 6.7-16.0 g/100 g, respectively [157]. As it could be seen from Saúl García-Pérez *et* al. results, the extremely high CO_2 flow rate (~14 L/min) and high amount of ethanol modifier (16-32%) increases yield recovery by 4.4-6.9-fold, as compared to our study results at similar SFE-CO_2 conditions. The significant positive effect of extraction temperature is also exemplified in the research of Saúl García-Pérez and co-workers: at the constant pressure of 10 MPa, the increase in

temperature by 20°C and 2-fold higher ethanol amount improves yield recovery by 37%. Because higher temperature increases solubility of analytes and reduces viscosity of solvent and analytes diffusion to the solvent is improved, increased temperature can easily disrupt the strong solute–matrix interactions caused by various bonds attractions and remove the solute from matrix easier. In the studies with *Rosmarinus officinalis* , also known as rosemary (belongs to the same *Lamiaceae* plant family as oregano), by Vázquez et al., the applied SFE-CO_2 conditions were milder (50°C, 20 and 30 MPa) than in our study, but the duration of extraction was by 90 min longer (in total, 5 hours), which resulted in 2-3 times higher non-polar fraction recovery (3.14 and 4.45 g/100 g at 20 and 30 MPa, respectively) [159].

In addition to SFE-CO_2, oregano sample was fractionated utilizing PLE with hexane at the following extraction conditions (**Table 13**): pressure 10.3 MPa, temperature 50°C, time 15 min (3 cycles x 5 min). Under these conditions, 3.08 g/100g DW of non-polar constituents, which was similar to Soxhlet (samples of 0.2-1 particle sizes) and SFE-CO_2 (+5% EtOH) results at 50°C. In 2013, PLE extraction of rosemary with hexane was carried out by *Vázquez* and others, applying the same pressure (10 MPa), however higher temperatures (100 and 150°C) and shorted extraction time (10 min). Rosemary samples yielded 9.87 g/100 g at 100°C and 15.63 g/100 g at 150°C; in both cases this is significantly higher than in our study: 2 times higher temperature resulted 3.2-fold higher non-polar constituents recovery, 3 times higher temperature resulted 5-fold higher yield.

As it could be seen comparing extractions yields under different extraction techniques (**Table 13**), temperature, pressure and time have a major impact in process efficiency. For example, if extraction is operated at the atmospheric pressure, the yield recovery remains high only at elevated extraction temperatures, otherwise yield significantly decreases (e.g. Soxhlet versus SLE). If temperature is medium and the pressure is high, yield recovery also is relatively high (in SFE-CO_2 extraction at 50°C with ethanol modifier). On the one hand, comparing all tested conventional and modern (high pressure) extractions techniques, PLE could be recommended for non-polar compound isolation from oregano. Firstly, PLE at 50°C takes only 15 minutes to obtain the same amount of hexane-soluble constituents, as compared to Soxhlet extraction, which is conducted at ~20°C higher temperature for 24-fold longer time. Although Soxhlet extraction is considered as the cheapest extraction technique, the significant reduction in extraction time and temperature could compensate higher cost of PLE. Secondly, applied pressure is not so high as in the case of SFE-CO_2 (10 MPa versus 45 MPa in SFE-CO_2), which could reduce the degradation of heat-labile compounds due to the high pressure and temperature combinations. On the other hand, the advantage of SFE-CO_2 against solvent-utilizing extraction techniques, like PLE, are the following: CO_2 gas is ideal since it is neither toxic nor explosive and is easy to remove

from the extracted products [4,76]. Furthermore, extracts obtained by SFE-CO_2 can be regarded as all natural and allowed for food application, also have GRAS status. Yet, CO_2 is a lipophilic solvent and that limits extraction performance by polarity. SFE-CO_2 yields higher than 2.8 g/100 g DW can be achieved by the addition of EtOH (total consumption of ~50 mL during 210 min of extraction, however increases the price of the extraction process itself. Either, high critical pressure is one of the biggest disadvantages of this kind of extraction [160]. Due to, for further solid residues utilizing after SFE-CO_2 was chosen PLE with polar solvents such as acetone, ethanol, water, ethanol/water mixture to obtain higher polar yield of plant material.

3.3. Isolation of polar constituents by conventional extractions methods and pressurized liquid extraction (PLE) optimization

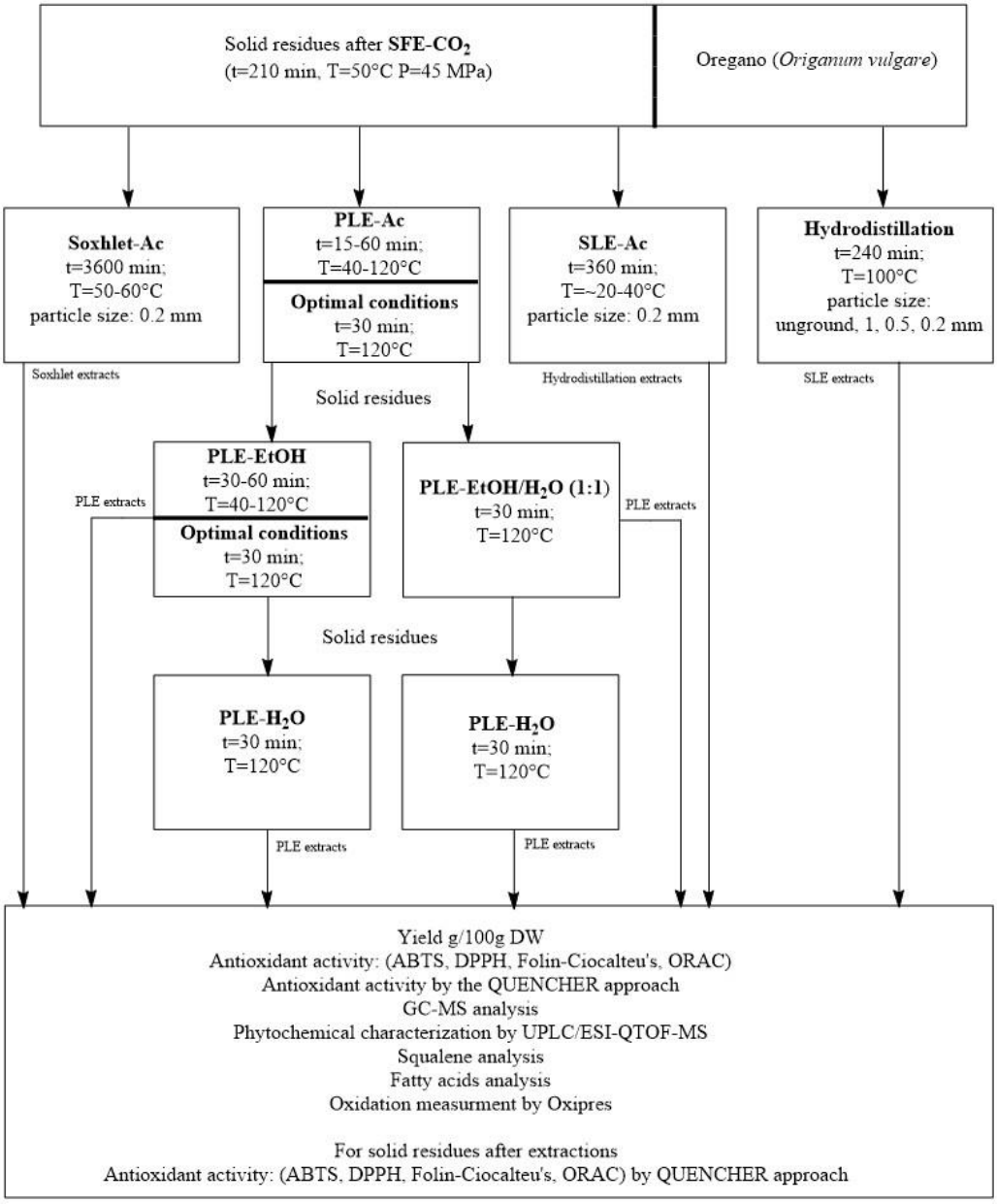

Fig. 15 Polar extracts isolation scheme

After SFE-CO_2 extraction in which non-polar (in case with EtOH modifier semi-polar) constituents are isolated, in plant solid residues could be left much more polar analytes, which could be extracted using different types of extraction.

Thus, solid residues after SFE-CO_2 at chosen conditions (50°C, 45 MPa, 210 min) were used in further extractions such as conventional extraction methods as SLE (using solvent acetone), Soxhlet extraction (using solvent acetone) and high pressure extraction PLE (using solvent acetone, and then step by step EtOH, EtOH/H_2O mixture (1:1), H_2O). Furthermore, not only essential oils but also water-soluble extracts were obtained after hydrodistillation. Extractions parameters are presented in **Fig. 15.**

3.3.1. Optimization of PLE parameters and model analysis

As it could be seen from non-polar extractions PLE extraction is significantly more effective, because during the shorter time could be reach the same or higher extractions yield, and also in some cases solvent amounts is lower than in others. For pressurized liquid extraction, broad range of solvents could be used: hexane, methanol, ethanol, acetone, water. However, PLE efficiency depends on many variables: temperature, time, pressure, solvent, plant material, so process optimization is the key to reach higher response factors values. For PLE optimization, residues of oregano after SFE-CO_2 (50°C, 45 MPa, 210 min) and acetone was used. Central composite design (CCD) and response surface methodology (RSM) were employed to study the effect of two independent variables on: (1) total extract yield (g/100 g DW); (2) total phenolic content (TPC, mg GAE/g of solid residue after SFE-CO2) and TPC/browning: time (15-60 min) and temperature (40-120°C).

3.3.1.1. Model analysis for PLE extraction yield

As presented in Table 13, extraction yields varied from 2.3 g/100 g DW to 9.1 g/100 g DW. The adequacy of the model was evaluated by the total determination coefficient (R^2) value of 0.9871, indicating a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2) of 0.9779 was in agreement with the predicted coefficient of determination (R^2) of 0.9321. Model evaluation is presented in the analysis of variance. The significance of each factor was determined using the Student test (p-value). The analysis of the quadratic regression models for extract yield showed that the model was significant ($p < 0.05$) with a F-value of 107.42 and the lack of fit is not significant to the pure error with a p value of 0.3690. The model shows that the factor with the higher effect on extract yield was the extraction temperature ($p < 0.0001$) followed by weaker effect of extraction time.

Table 14. Oregano PLE parameters by CCD, predicted and actual values of PLE extracts yields, TPC amount in extracts and TPC/browning ratio

Run	Parameters		Yield, g/100 g		TPC, mg GAE/g		TPC/browning	
	Time, min	*Temperature °C*	*Actual values*	*Predicted values*	*Actual values*	*Predicted values*	*Actual values*	*Predicted values*
1	37.5	80	4.94±0.15^{bcd}	5.12	9.79±0.27^{d}	10.12	1.8	1.88
2	37.5	80	5.50±0.46^{d}	5.12	10.91±0.14^{e}	10.12	2.11	1.88
3	15.0	40	2.26±0.08^{a}	2.14	2.82±0.01^{a}	2.42	0.83	0.84
4	37.5	80	4.85±0.06b^{c}	5.12	9.54±0.07^{d}	10.12	1.84	1.88
5	60.0	120	8.36±0.01^{ef}	8.63	20.44±0.27^{g}	21.31	3.96	4.03
6	37.5	80	4.79±0.44^{bc}	5.12	9.51±0.04^{d}	10.12	1.7	1.88
7	60.0	80	5.30±0.13^{cd}	4.97	11.31±0.08^{f}	9.70	2.35	2.10
8	15.0	120	7.96±0.09^{e}	8.05	23.14±0.07^{h}	22.69	3.82	3.72
9	15.0	80	4.35±0.04^{b}	4.38	8.12±0.06^{c}	9.15	1.58	1.67
10	60.0	40	2.66±0.02^{a}	2.72	3.98±0.02^{b}	4.72	1.22	1.40
11	37.5	120	9.14±0.15^{g}	8.78	23.1±0.01^{h}	22.69	3.84	3.87
12	37.5	80	5.21±0.03^{cd}	5.12	10.28±0.01^{c}	10.12	1.78	1.88
13	37.5	40	2.82±0.27^{a}	2.88	4.34±0.02^{b}	4.18	1.31	1.13
				Optimal conditions				
	30	**120**	**8.87±0.22^{fg}**	**8.63**	**30.60±0.07^{i}**	**22.84**	**4.20**	**4.09**
		Difference:	**+2.8%**		**+34.0%**		**+2.7%**	

Referred average values of three determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

The model shows that temperature and the square of the temperature ($p < 0.05$) is significant model terms for extraction yield. Time is not significant for this response factor ($p > 0.05$).

Table 15. Model analysis for PLE extracts yield

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	54.34	5	10.87	107.42	<0.0001*
τ-time	0.51	1	0.51	5.05	0.0595**
T-temperature	52.33	1	52.53	517.31	<0.0001*
τT	0.000	1	0.000	0.000	1.0000**
τ^2	0.54	1	0.54	5.38	0.0534**
T^2	1.40	1	1.40	13.80	0.0075*
Residual	0.71	7	0.10		
Lack of Fit	0.36	3	0.12	1.38	0.3690**
Pure Error	0.35	4	0.087		
Cor Total	55.04	12			

*-significant; **-not significant

Predicted values of extract yield were calculated using the regression model and compared with experimental values. Second order polynomial regression model, which is an empirical relationship between dependent variables and the independent test variables (τ, T):

$$Yield, \frac{g}{100g/DW} = 5.12 + 0.29 * \tau + 2.95 * T + 0.000 * \tau * T - 0.44 * \tau^2 + 0.71 * T^2 \quad (7)$$

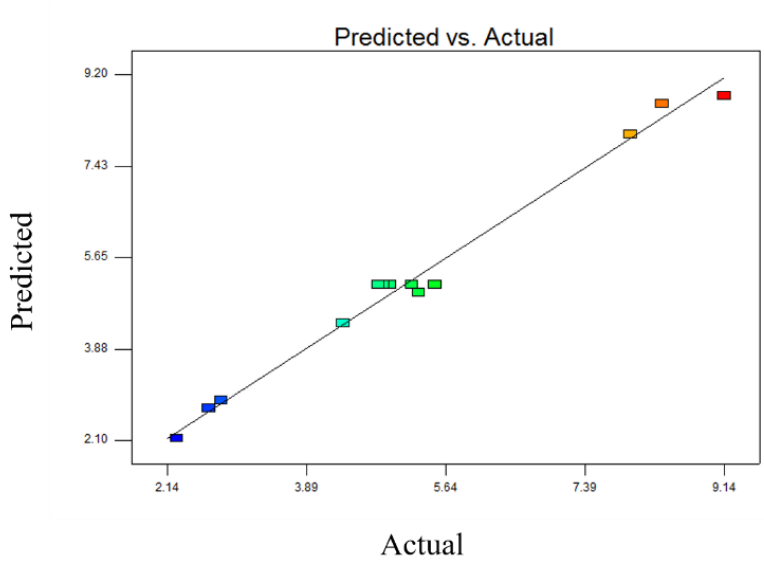

Fig. 16 Actual and predicted RSM values on oregano yield

Response surface plots showing the effect of extraction time and temperature on extraction yields are shown in Fig. Model graphs illustrates time and temperature effect on extraction yield at constant pressure (10.3 MPa). It is clearly seen, that temperature has a major impact on extraction yield, while time (shortest or highest time period) has no significant influence on

extraction yield. Also, it could be seen, that the longest extraction time have negative effect and leads extraction yield to lower values. Obtaining the highest extraction yield (9.1 g/100 g DW) optimal conditions are: time – 37.5 min, temperature – 120°C.

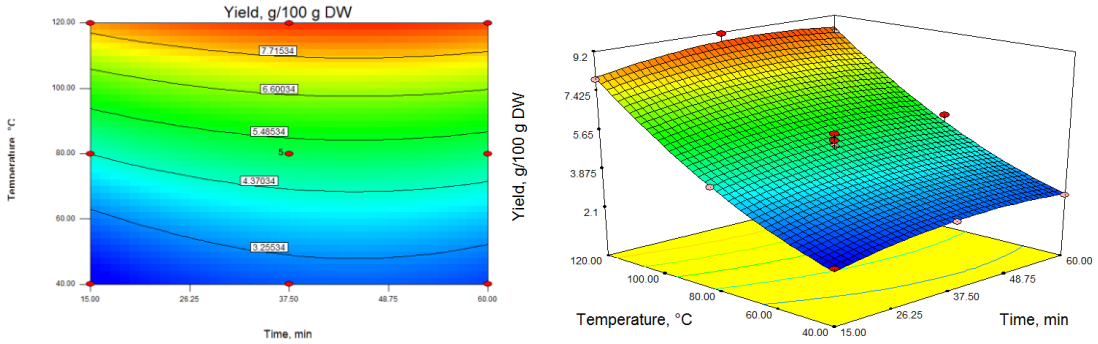

Fig. 17 3D RSM plots of the dependencies of oregano extraction yield on time and temperature

3.3.1.2. Model analysis for total phenolic content (TPC)

As presented in table TPC varied from 2.8 mg GAE/g DW to 23.1 mg GAE/g DW. The adequacy of the model was evaluated by the total determination coefficient (R^2) value of 0.9871, indicating a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2) of 0.9780 was in agreement with the predicted coefficient of determination (R^2) of 0.8940. Model evaluation is presented in the analysis of variance. The analysis of the quadratic regression models for TPC showed that the model was significant ($p < 0.05$) with a F-value of 107.55 and the lack of fit is not significant to the pure error with a p value of 0.0671. The model shows that the factor with the higher effect on TPC was the extraction temperature ($p < 0.0001$) followed by weaker effect of extraction time. The model shows that temperature and the square of the temperature ($p < 0.05$) is significant model terms for TPC. Time is not significant for this response factor ($p > 0.05$).

Table 16. Model analysis for PLE extracts total phenolic content (TPC)

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	549.62	5	109.92	107.55	<0.0001
τ-time	0.45	1	0.45	0.44	0.5
T-temperature	514.12	1	514.12	503.00	<0.0001
τT	3.72	1	3.72	3.64	0.0979
τ^2	1.33	1	1.33	1.30	0.2917
T^2	30.28	1	30.28	29.63	0.0010
Residual	7.15	7	1.02		
Lack of Fit	5.75	3	1.92	5.47	0.0671
Pure Error	1.40	4	0.35		
Cor Total	556.78	12			

*-significant; **-not significant

Predicted values of TPC were calculated using the regression model and compared with experimental values. Second order polynomial regression model, which is an empirical relationship between dependent variables and the independent test variables (τ, T):

$$TPC\ \frac{mg\ GAE}{g\ DW} = 10.12 + 0.28 * \tau + 9.26 * T - 0.96 * \tau * T - 0.69 * \tau^2 + 3.31 * T^2 \qquad (8)$$

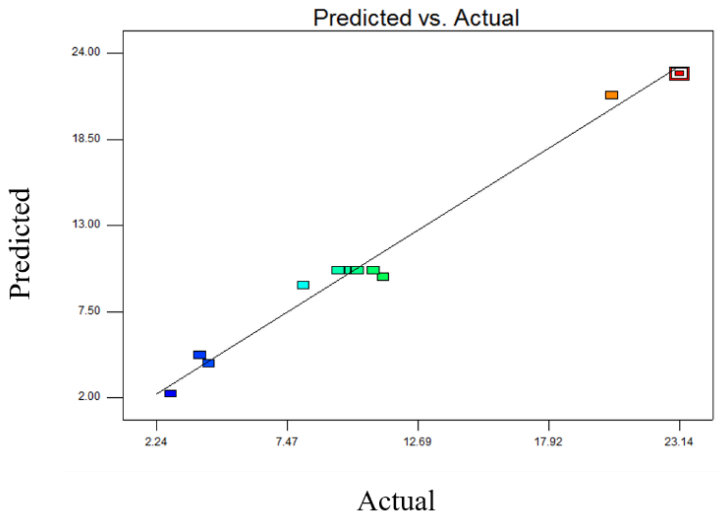

Fig. 18 Actual and predicted RSM values on oregano TPC

Response surface plots showing the effect of extraction time and temperature on TPC are shown in Fig. Model graphs illustrates time and temperature effect on TPC yield at constant pressure (10.3 MPa). Temperature has a major impact on TPC as well as in extraction yield, while time has no significant influence on extraction yield, but it is important to mark that the highest TPC obtained at higher temperature and the shortest time (15 min), and the longest time at highest temperature resulted 12% lower TPC. Considering obtained results optimal conditions to reach highest TPC (23.1mg GAE/g DW) are: time – 37.5 min, temperature – 120°C.

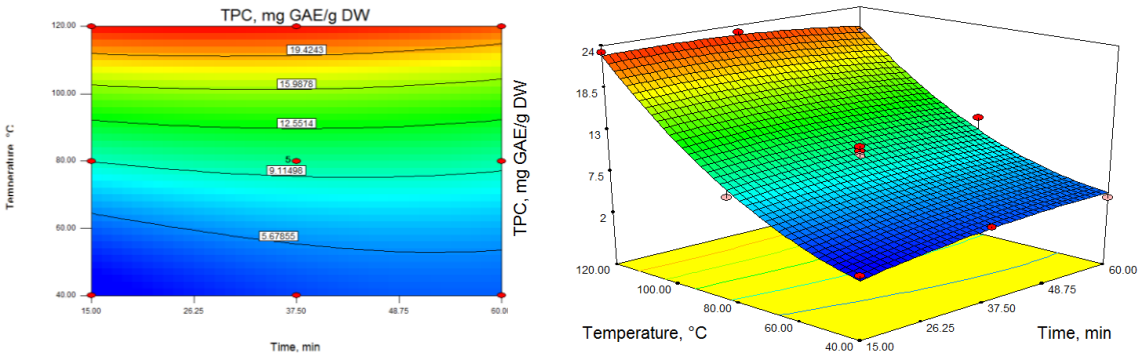

Fig. 19 3D RSM plots of the dependencies of oregano TPC on time and temperature

3.3.1.3. Model analysis for TPC/browning

Since PLE extractions were carried out with temperatures up to 120°C, it was decided that browning reactions could be involved. The absorbance at 420 nm of PLE extracts obtained at higher temperatures (80°C, 120°C) was higher than absorbance detected at 40°C extracts. The increase of TPC values of the extracts obtained at higher temperatures may be related to the production of melanoidins, reported to have antioxidant activity. However, melanoidins could change phenolic compounds profile, therefore it was decided to maximize TPC and browning ratio, because the higher TPC value, the lower browning value, the higher the ratio. As presented in **Table 14** TPC/browning ratio varied from 0.8 to 4. The adequacy of the model was evaluated by the total determination coefficient (R^2) value of 0.9803, indicating a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2) of 0.97663 was in agreement with the predicted coefficient of determination (R^2) of 0.8745. Model evaluation is presented in the analysis of variance. The analysis of the quadratic regression models for TPC/browning showed that the model was significant ($p < 0.05$) with a F-value of 69.85 and the lack of fit is not significant to the pure error with a p value of 0.2305. The model shows that the effect on higher TPC/browning value has both: time ($p < 0.0280$) and extraction temperature ($p < 0.0001$).

Table 17. Model analysis for total phenolic content and browning ratio

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	12.90	5	2.58	69.85	<0.0001*
τ-time	0.28	1	0.28	7.63	0.0280*
T-temperature	11.37	1	11.37	307.91	<0.0001*
τT	0.016	1	0.016	0.42	0.5362**
τ^2	6.43E-005	1	6.43E-005	1.743E-003	0.9679**
T^2	1.04	1	1.04	28.27	0.0011*
Residual	0.26	7	0.037		
Lack of Fit	0.16	3	0.054	2.20	0.2305**
Pure Error	0.098	4	0.024		
Cor Total	13.16	12			

Predicted values of TPC were calculated using the regression model and compared with experimental values. Second order polynomial regression model, which is an empirical relationship between dependent variables and the independent test variables (τ, T):

$$\frac{TPC}{browning} = 1.88 + 0.22 * \tau + 1.38 * T - 0.062 * \tau * T + 4.828E - 003 * \tau^2 + 0.61 * T^2 \quad (9)$$

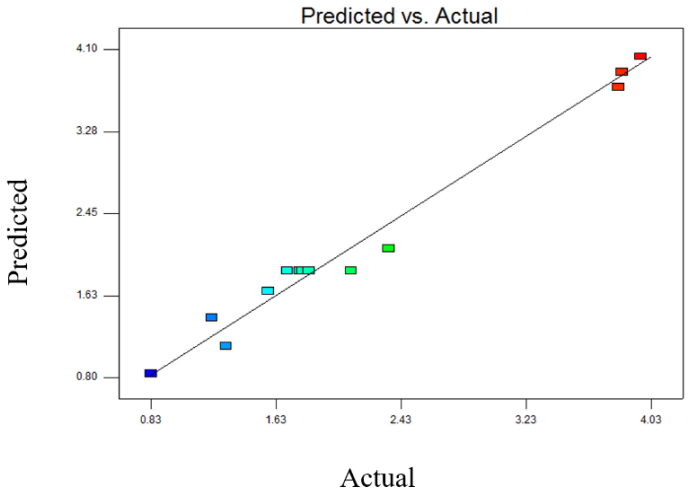

Fig. 20 Actual and predicted RSM values on TPC/browning

Response surface plots showing the effect of extraction time and temperature on TPC/browning are shown in Fig. Model graphs illustrates time and temperature effect on TPC/browning at constant pressure (10.3 MPa). Browning reactions causes phenolics oxidation and the loss of their good properties. As it is known browning reactions takes place at high temperatures. The higher ratio is the goal, because it means that TPC it is not strongly effected by browning reactions. Temperature and time has an impact on TPC/browning. Longer time at highest temperature resulted highest TPC/browning ratio, because at this point high TPC was obtained, also high ratio values obtained at same temperature, but a shorter time of period, as well as the shortest time period. Considering obtained results at all three response factors – yield, TPC, TPC/browning, optimal conditions to reach highest values is: time – 37.5 min, temperature – 120°C.

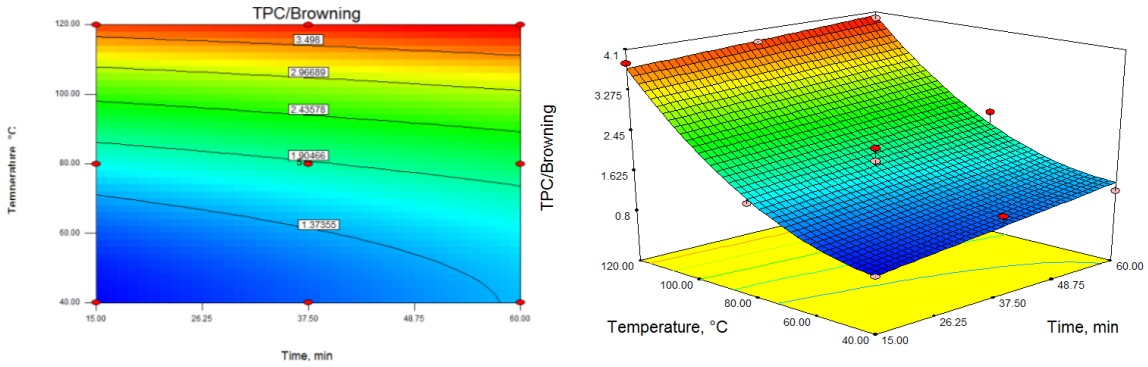

Fig. 21 3D RSM plots of the dependencies of oregano TPC/browning yield on time and temperature

After optimization, when optimal conditions were established PLE extraction was carried out with EtOH, H_2O and EtOH/H_2O (1:1) mixture to obtain maximum polar constituents recovery.

3.3.2. Polar and semi-polar constituents isolation by conventional and high pressure extractions

Polar extracts as well as non-polar extracts could be obtained by different extractions and different solvents. Extraction yields, expressed as g/100 g DW of plant material, under the different extraction conditions are reported **Table 18.** After hydrodistillation there were got not only essential oil fractions, but also received aqueous phase (hydrolats) fractions. For essential oil isolation were used unground and ground (0.2, 0.5, 1 mm) fractions plant material, so and hydrolats were obtained from the same four fractions. Polar fraction yield from unground oregano was the highest (19.11 g/100g DW), while extraction effectiveness was significantly decreased by 25-33% reducing particle size of plant material up to smaller particles – 0.2, 0.5, 1 mm (14.33g/100 g DW, 13.18 g/100g DW, 12.85 g/100g DW, respectively). The water-soluble fraction yield from differently ground samples did not differ significantly. Smaller particle size fractions have higher surface area and more polar constituents should be extracted from them, but during hydrodistillation plant material all extraction time is exposed to water at its boiling point, so isolated constituents could degrade until the end of extraction.

Other two conventional methods used for polar constituents isolation – Soxhlet extraction and SLE. For both extractions were used the same solvent – acetone and the time was set the same – 360 min. Soxhlet extraction yielded 6.28 g/100g DW of acetone-soluble analytes, SLE isolation efficiently was 3-3.9-fold lower (significantly not differed, on average 1.85 g/100g DW). There is two main reasons why Soxhlet extract is way more effective: temperature, and the extraction process. Temperature in Soxhlet extraction was 56°C (acetone boiling point) and in the SLE temperature was ~20°C (room temperature) and 40°C, and as mentioned in non-polar constituents extractions, the higher the temperature, the higher the yield. During the Soxhlet extraction, plant material in extractor is filled with condensed solvent, which condenses in the condenser from distillation flask. When the solvent in extractor reaches the overflow level, solvent through the trap goes back to distillation flask. In flask, isolated constituents are separated from solvent by distillation method and solvent fills extractor again. This step is repeated until extraction is finished. In the SLE solvent and plant material all extraction time are kept together, no flow movements are involved. Pizzale and coworkers (2002) applied SLE with methanol. Dried *Origanum onites* were extracted 24h with methanol (+1 h refluxed). Depending on which anatomical part was used for extraction, methanol-soluble extracts yield varied: from flowers plant material yield varied from 14.8 g/100g DW to 18.9 g/100g DW; from leaves plant material yield varied from 20.3 g/100g DW to 22.5 g/100g DW [161].

After PLE optimization with acetone, for further steps other solvents were used, but first step before all solvents used was SFE-CO_2 plant material extract with PLE using acetone at optimal conditions. Acetone-soluble constituents yielded 8.70 g/100 g DW (and that amount is similar and statistically not different from analytes amount obtained with conventional Soxhlet extraction using acetone). With other solvents, ethanol-soluble, water-soluble constituents isolation efficiently increased as follows: PLE-Ac-EtOH/H_2O-H_2O (4.48 g/100g DW) < PLE-Ac-EtOH (40°C, 30min, 7.14 g/100g DW) < PLE-Ac-EtOH (120°C, 30min, 15.13 g/100 g DW) < PLE-Ac-EtOH (120°C, 60min, 16.67 g/100g DW) < PLE-Ac-EtOH-H_2O (22.27 g/100g DW) < PLE-Ac-EtOH/H_2O (1:1) (28.99 g/100g DW). The highest polar extract yield was obtained after PLE at optimal conditions with EtOH/H_2O mixture (1:1) (28.99 g/100 g DW). These constituents yields shows only how much of them were obtained using either ethanol or water, or their mixture, but if each step of extraction would be presented as one, extractions efficiently would be way more higher: PLE-Ac-EtOH/H_2O-H_2O totally yielded 42.17 g/100 g DW, PLE-Ac-EtOH (40°C, 30min) – 15.84 g/100g DW, PLE-Ac-EtOH (120°C, 30min) – 23.83 g/100g DW, PLE-Ac-EtOH (120°C, 60min) – 25.37 g/100g DW, PLE-Ac-EtOH-H_2O – 46.1 g/100g DW, PLE-Ac-EtOH/H_2O (1:1) – 37.69 g/100g DW. Plaza *et* all (2010) reported results obtained with subcritical water PLE: extraction took 20 min, there was set two temperatures: 100°C and 200°C, pressure 10 MPa. In different temperatures yields varied: ~15g/100 g DW at 100°C and ~ 40g/100g DW at 200°C [162]. Miron and coworkers (2011) reported PLE yields obtained using solvents water, ethanol and ethanol/water (1:1) [124]. Extraction time was set 20 min, pressure – 10.3 MPa, for water and ethanol temperatures were between 100-150°C, for ethanol/water (1:1) - 100°C. With water extraction yields were ~33 g/100 g DW and ~50 g/100 g DW, with ethanol ~ 12g/100 g DW and ~20 g/100 g DW, respectively. PLE with ethanol/water mixture (1:1) yielded ~32 g/100 g DW. Also, in this study was obtained, that raising temperature to 200°C can increase yield recovery 1.2-1.8-fold with solvent water, and 2-3.3-fold with solvent ethanol. Herrero et al. (2010) reported results obtained by the same PLE extraction conditions but as plant material was used rosemary.

Yield at 100°C with solvent water was 24.0 g/100 g DW, at 150°C – 37.3 g/100 g DW. With ethanol at 100°C yield recovery was 22.9 g/100 g DW, at 150°C – 29.1 g/100 g DW [163].

Table 18. Conventional and high pressure extractions parameters and isolated polar and semi-polar constituents (hydrodistillation, Soxhlet, SLE, SFE-CO_2, PLE) yields, expressed as g/100g sample and g/100g DW.

Sample	Particle size	Extractions parameters			Extract yield		Solid residues
	mm	*Pressure, MPa*	*Temperature, °C*	*Time, min*	*g/100 g*	*g/100 g DW*	*g/100 g DW*
Conventional extractions							
HD	unground		100	240	-	19.11±0.04^{ef}	80.57±0.04^{d}
HD	1	-	100	240	-	12.85±0.79^{d}	86.88±0.79^{f}
HD	0.5	-	100	240	-	13.18±3.53^{d}	86.59±3.53^{ef}
HD	0.2	-	100	240	-	14.33±3.51^{d}	85.47±3.51^{ef}
Soxhlet-Ac	0.2	-	56	360	6.28±0.14^{bc}	6.15±0.14^{bc}	91.79±0.14^{f}
SLE-Ac	0.2	-	40	360	2.08±0.04^{a}	2.04±0.04^{a}	95.95±0.04^{g}
SLE-Ac	0.2	-	R.T. (~20)	360	1.62±0.06^{a}	1.58±0.06^{a}	96.41±0.06^{g}
High pressure fractionation							
PLE-Ac	0.2	10.3	120	30	8.87±0.22^{c}	8.70±0.22^{c}	89.34±0.22^{f}
PLE-Ac-EtOH	0.2	10.3	120/40	30/30	7.29±0.22^{b}	7.14±0.21^{bc}	82.20±0.01^{de}
PLE-Ac-EtOH	0.2	10.3	120/120	30/30	15.46±0.28^{d}	15.13±0.27^{d}	74.19±0.06^{c}
PLE-Ac-EtOH	0.2	10.3	120/120	30/60	17.02±0.04^{e}	16.67±0.04^{de}	72.66±0.26^{c}
PLE-Ac-EtOH/H_2O (1:1)	0.2	10.3	120/120	30/30	29.52±0.43^{g}	28.99±0.42^{g}	60.40±0.20^{b}
PLE-Ac-EtOH-H_2O	0.2	10.3	120/120/120	30/30/30	22.72±0.46^{f}	22.27±0.44^{f}	51.92±0.51^{a}
PLE-Ac-Et/H_2O (1:1)- H_2O	0.2	10.3	120/120/120	30/30/30	4.58±0.02^{a}	4.48±0.02^{ab}	55.92±0.18^{a}

Referred average values of three determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

In this study for PLE was selected lower temperature (120°C) than the maximum selected temperature in discussed reported studies and this was the result of higher obtained yields with solvent water and ethanol, with solvent ethanol/water yields recovery were almost the same. As it mentioned before, in this study PLE was carried out step by step, and by that reason extracts yield with same solvents could be the same or higher. Glisic et al (2010) isolated polar constituents from *Salvia officinalis* (also known as sage, belonging to *Lamicetae* family as oregano) with Soxhlet extraction for 4 hours with ethanol/water mixture (70:30 by volume). Obtained constituents yielded 26.5 g/100 g DW [164]

It is clear that the highest polar extracts yield could be reached with water, ethanol or ethanol/water mixture. The best extraction in order to get polar extracts would be PLE extraction. It takes less time than others, and in comparison, with different solvents PLE extraction shows better yield recovery than others. Comparing PLE extraction with water and hydrodistillation extraction which is carried with the same solvent – water, the yield obtained after PLE extraction is 1.2-1.8-fold higher, than after hydrodistillation. It is important to mark that PLE extraction in this case takes 90 minutes, while hydrodistillation takes almost three times longer – 240 minutes. Also for hydrodistillation much more distilled water amount is needed. PLE extraction could be carried out with about 3 times smaller water quantities. It is much more convenient when it comes to preparation of extracts for further storage. Aqueous extracts should be lyophilized before storage, so the less water was used, the faster extracts could be lyophilized. Comparing three different extractions – Soxhlet extraction, PLE, SLE – with the same solvent – acetone PLE extraction also is superior. In Soxhlet extraction the yield is similar to yield obtained after PLE extraction, but as mentioned earlier Soxhlet extraction takes 360 minutes to reach that yield, when in PLE extraction it takes only 30 minutes (and the yield is a little bit higher, but not significantly). Also, less acetone is needed. SLE is practically useless, because yield recovery reaches only ~2g/100g DW, which is 3-4 times smaller than in Soxhlet and SLE takes the same time as Soxhlet extraction, and the solvent amount is the same. So, the best extraction for polar extracts, using different solvents would be PLE extraction. It saves solvents, time and gives higher yields.

3.4. In vitro antioxidant capacity of non-polar and polar oregano extracts and plant material

Scavenging of different types of reactive oxygen and nitrogen species, mostly free radicals is thought to be one of the main mechanisms of the antioxidant action exhibited by phenolic phytochemicals. In the assessment of radical-scavenging properties, both synthetic and biologically-relevant free radicals are used. The synthetic nitrogen-centered $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals are not biologically relevant, but are often used as indicator compounds in testing hydrogen-donation capacity and thus antioxidant activity [165]. In vitro antioxidant activity of all

obtained extracts after all extractions were evaluated as total phenolic content (mg GAE/g sample; mg GAE/g DW), $ABTS^{\bullet+}$ (mg TE/g sample; mg TE/g DW), $DPPH^{\bullet}$ (mg TE/g sample; mg TE/g DW), ORAC (mg TE/g sample; mg TE/g DW).

3.4.1. Total phenolic content (TPC) by Folin-Ciocalteu's of oregano extracts

Many available methods of quantification of total phenolic content in food products are based on the reaction of phenolic compounds with a colorimetric reagent, which allows measurement in the visible portion of the spectrum.

Table 19. Total phenolic content (TPC) of non-polar and polar oregano extracts, isolated by different conventional and high pressure extraction techniques, expressed as mg GAE/g extract and mg GAE/g DW

Extract	Extraction conditions	TPC by Folin-Ciocalteu's	
		mg GAE/g extract	*mg GAE/g DW*
Non-polar extracts			
Conventional extractions (from starting plant material):			
Soxhlet-He-ungroud	69°C, 360 min	27.94 ± 0.54^{ab}	0.66 ± 0.01^{abc}
Soxhlet-He-1 mm	69°C, 360 min	25.59 ± 0.91^{a}	0.77 ± 0.03^{abcd}
Soxhlet-He-0.5 mm	69°C, 360 min	24.51 ± 0.73^{a}	0.80 ± 0.02^{abcd}
Soxhlet-He-0.2 mm	69°C, 360 min	27.07 ± 0.94^{a}	0.87 ± 0.03^{abcd}
SLE-He	50°C, 360 min	32.16 ± 0.75^{bc}	0.57 ± 0.01^{abc}
High pressure extractions (from starting plant material):			
SFE-CO_2	25°C, 210 min, 45 MPa	37.86 ± 1.19^{e}	0.50 ± 0.02^{ab}
SFE-CO_2	40°C, 210 min, 45 MPa	25.59 ± 0.44^{a}	0.39 ± 0.01^{a}
SFE-CO_2	50°C, 210 min, 45 MPa	24.51 ± 0.66^{a}	0.49 ± 0.01^{ab}
SFE-CO_2	70°C, 210 min, 45 MPa	36.29 ± 1.43^{cde}	0.83 ± 0.03^{abcd}
SFE-CO_2 (5% EtOH)	40°C, 210 min, 45 MPa	33.40 ± 1.12^{cd}	0.98 ± 0.03^{bcde}
SFE-CO_2 (5% EtOH)	50°C, 210 min, 45 MPa	39.81 ± 1.61^{e}	1.25 ± 0.05^{def}
SFE-CO_2 (5% EtOH)	70°C, 210 min, 45 MPa	37.34 ± 1.47^{de}	1.03 ± 0.04^{cde}
SFE-CO_2 (after HD)	70°C, 210 min, 45 MPa	59.61 ± 2.30^{f}	1.19 ± 0.05^{ef}
PLE-He	50°C, 15 min, 10 MPa	55.38 ± 0.86^{f}	1.71 ± 0.03^{f}
Polar extracts			
Conventional extractions (from starting plant material):			
HD-Unground	100°C, 360 min	376.84 ± 2.21^{o}	72.01 ± 0.42^{r}
HD-1 mm	100°C, 360 min	257.48 ± 1.86^{k}	33.09 ± 0.24^{k}
HD-0.5 mm	100°C, 360 min	228.25 ± 6.83^{i}	30.08 ± 0.90^{o}
HD-0.2 mm	100°C, 360 min	235.77 ± 1.58^{j}	33.79 ± 0.33^{l}
Conventional extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
SLE-Ac	40°C, 360 min	132.36 ± 1.78^{g}	2.75 ± 0.04^{g}
SLE-Ac	R. T, 360 min	144.67 ± 2.68^{h}	2.29 ± 0.04^{g}
Soxhlet-Ac	56°C, 360 min	59.74 ± 1.72^{f}	3.75 ± 0.11^{h}
High pressure extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
PLE-Ac	120°C, 30 min, 10 MPa	356.26 ± 0.85^{m}	30.99 ± 0.07^{j}
PLE-Ac-EtOH	40°C, 30 min, 10 MPa	370.22 ± 0.49^{n}	56.01 ± 0.07^{o}
PLE-Ac-EtOH	120°C, 30 min, 10 MPa	423.56 ± 0.85^{p}	70.61 ± 0.17^{p}
PLE-Ac-EtOH	120°C, 60 min, 10 MPa	494.00 ± 2.15^{r}	35.27 ± 0.15^{m}
PLE-Ac-EtOH/H_2O (1:1)	120°C, 30 min, 10 MPa	306.83 ± 0.65^{l}	88.95 ± 0.19^{s}
PLE-Ac-EtOH-H_2O	120°C, 30 min, 10 MPa	231.76 ± 0.52^{ij}	51.61 ± 0.12^{n}
PLE-Ac-Et/H_2O-H_2O	120°C, 30 min, 10 MPa	56.97 ± 0.23^{f}	2.55 ± 0.01^{g}

Referred average values of five determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

The Folin–Ciocalteu's assay is this kind of method and has been proposed as a standardized method for measurement of antioxidant capacity of food products and dietary supplements. The

F–C assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectroscopically at approximately 760 nm. Although the exact chemical nature of the Folin-Ciocalteu's reaction is unknown, it is believed that sequences of reversible one- or two-electron reduction reactions lead to blue species [166,167,168]. Total phenolic content of non-polar and polar extracts, of oregano, obtained by conventional and high pressure extraction techniques, are presented in **Table 19**. TPC values in all obtained extracts varied from 24.5 mg GAE/g extract to 494 mg GAE/ g extract, corresponding to 0.8 mg GAE to 35.3 mg GAE/g DW. Among non-polar extracts TPC values varied from 24.5 mg GAE/g extract to 59.6 mg GAE/g extract (0.8 mg GAE/g DW – 1.7 mg GAE/g DW). Total phenolic content values in extracts obtained by conventional extractions methods (Soxhlet and SLE) varied in small range from 24.5 mg GAE/g extract to 31.2 mg GAE/g extract. With high pressure extractions (SFE-CO_2 and PLE) extracts TPC values were higher up to 59.6 mg GAE/g extract (1.2 mg GAE/g DW), except values obtained at SFE-CO_2 at 40°C and 50°C (these values were similar to values corresponded at Soxhlet extraction and statistically not differed). Highest TPC value among non-polar extracts had hexane-soluble extract isolated by PLE.For non-polar extracts, with highest total phenolic content values, isolation the most effective extraction would be PLE using solvent hexane. As could be seen extract after SFE-CO_2 with plant material after hydrodistillation had the highest TPC value, besides that value obtained in hexane-soluble PLE extracts statistically not differed from this value, and it should be noted, that for PLE extraction was used milder conditions (50°C, 10 MPa, while in this particular SFE-CO_2 extraction - 70°C, 45 MPa). Most important that PLE took only 15 minutes and in comparison, with conventional extraction methods time was 24-fold shorter and obtained total phenolic content values was up to 2-fold higher. Polar extracts (obtained by conventional extraction methods) TPC values varied from 60 mg GAE/g extract to 257.5 mg GAE/g extract. Lowest TPC value had acetone-soluble extract obtained Soxhlet extraction, while in SLE obtained values (extraction took the same time – 360 min, and lower temperatures – R. T. and 40°C) was more than 2-fold higher. In water-soluble hydrodistillation extracts TPC values varied in large range and in different particle size fractions was statistically different, but the highest one obtained in extract obtained from unground fraction. In extracts obtained by high pressure extractions (with different solvents) values varies from 56.9 mg GAE/ g extract to 494 mg GAE/g extract. Lowest value was obtained in extract which was isolated from plant material, which prior was extracted with acetone, EtOH/H_2O mixture (1:1) and finally with H_2O (60 mg GAE/g extract). As could be seen from **Table 19** some values were similar in those obtained by conventional extraction methods, nevertheless for high pressure extraction shorter time was required to obtain similar and higher TPC values. Furthermore, after hydrodistillation there a lot of water-soluble extract should

be lyophilized before further uses and it also takes a lot of time, while after PLE isolated water-soluble extract amount is smaller and faster could be prepared for the further use. In previous reported data different authors obtained different results. Dambolena et al. (2010) studied different oregano species for its total phenolic content. For research was used different oregano species: *Origanum vulgare spp. virens* (Hoffm. et Link) letswaart, *Origanum x applii* (Domin) Boros, *Origanum x majoricum* Cambess, and *O. Vulgare L. spp. vulgare*. Extracts were prepared by mixing plant material with deionized water and left it stir for 24 hours at room temperature. Total phenolic content in different samples varied from 8.9 mg GAE/g DW to 19.4 mg GAE/g DW [51]. Baranauskienė et al. (2013) investigated TPC in water-soluble oregano extracts obtained after hydrodistillation. The highest obtained TPC yields was in water-soluble extract obtained from oregano at full flowering period (330 mg GAE/ g sample) at butonization period TPC value was lower – 269 mg GAE/ g sample [169]. Texeira and coworkers (2013) investigated total phenolic content in extracts obtained by different conditions: hot water extract – plant material was soaked for 3 hours with hot (boiling) water, cold water extract – plant material was soaked for 3 days at room temperature water, ethanol extract – plant material was soaked for 3 days at room temperature with ethanol. Highest TPC value obtained in hot water extract – 17.8 mg GAE/ g sample, lower in the ethanolic extract – 13.5 mg GAE/g sample, and the lowest in cold water extract – only 6.4 mg GAE/g sample [57]. Zheng and Wang (2001) carried out study in which established total phenolic content in fresh Greek oregano (*Origanum vulgare ssp. Hirtum*) extract (fresh plant material was extracted with phosphate buffer). Obtained TPC value was 11.8 mg GAE/g FW [170]. Ünver et al. (2009) determined TPC in dried *Origanum vulgare* which extract was isolated by soaking plant material with 90% methanol + 9% water + 1% acetic acid at 24°C for 24 hours. Obtained TPC value was 420.5 mg GAE/g sample [171]. Rodrıguez-Meizoso and coworkers (2006) isolated constituents from oregano leaves by subcritical water extraction at different temperatures (25-200°C) and times (15-30min). TPC values varied from 0.08 to 0.18 mg GAE/ mg extract (lowest values obtained in extracts at higher temperatures 100-200°C and shortest time – 15 min, highest values obtained at 50°C, at this temperature values at 15min and 30 min was similar) [3]. As it could be seen there is a lot of factors from which depends total phenolic content in isolated extracts. Temperature, pressure, solvent has a big impact on results. Also, as could be seen from discussed data, not only from extractions conditions, but also from selected plant material (for example its growth stage) depends total phenolic content.

3.4.2. Radical scavenging capacity of isolated non-polar and polar extracts

For isolated extracts, radical scavenging capacity was measured applying two most common methods: $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. The ABTS cation radical ($ABTS^{\bullet+}$) which absorbs at 734 nm

(giving a bluish-green colour) is formed by the loss of an electron by the nitrogen atom of ABTS (2,2'-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid)). In the presence of Trolox (or of another hydrogen donating antioxidant), the nitrogen atom quenches the hydrogen atom, yielding the solution decolorization [167,168].

Table 20. ABTS scavenging assay values obtained in various non-polar and polar extracts isolated by different conventional and high pressure extraction techniques, expressed as mg TE/g extract and mg TE/g DW

Extract	Extraction conditions	$TEAC_{ABTS}$	
		mg TE/g sample	*mg TE/g DW*
Non-polar extracts			
Conventional extractions (from starting plant material):			
Soxhlet-He-ungroud	69°C, 360 min	20.92±0.76^{a}	0.52±0.02^{a}
Soxhlet-He-1 mm	69°C, 360 min	17.85±0.32^{a}	0.54±0.01^{a}
Soxhlet-He-0.5 mm	69°C, 360 min	19.86±0.16^{a}	0.65±0.01^{a}
Soxhlet-He-0.2 mm	69°C, 360 min	21.01±0.71^{a}	0.68±0.02^{a}
SLE-He	50°C, 360 min	386.11±3.96^{f}	6.87±0.07^{cd}
High pressure extractions (from starting plant material):			
SFE-CO_2	25°C, 210 min, 45 MPa	18.95±0.45^{a}	0.25±0.01^{a}
SFE-CO_2	40°C, 210 min, 45 MPa	16.44±0.75^{a}	0.25±0.01^{a}
SFE-CO_2	50°C, 210 min, 45 MPa	22.23±0.76^{a}	0.52±0.02^{a}
SFE-CO_2	70°C, 210 min, 45 MPa	24.47±1.19^{a}	0.56±0.03^{a}
SFE-CO_2 (5% EtOH)	40°C, 210 min, 45 MPa	26.18±1.74^{a}	0.76±0.05^{a}
SFE-CO_2 (5% EtOH)	50°C, 210 min, 45 MPa	32.09±1.25^{a}	1.00±0.04^{a}
SFE-CO_2 (5% EtOH)	70°C, 210 min, 45 MPa	61.13±1.70^{b}	1.69±0.05^{a}
SFE-CO_2 (after HD)	70°C, 210 min, 45 MPa	111.60±5.17^{c}	2.23±0.12^{ab}
PLE-He	50°C, 15 min, 10 MPa	480.52±4.21^{g}	14.80±0.13^{e}
Polar extracts			
Conventional extractions (from starting plant material):			
HD-Unground	100°C, 360 min	940.13±11.40^{l}	179.66±2.18^{n}
HD-1 mm	100°C, 360 min	867.51±17.65^{k}	111.47±2.27^{i}
HD-0.5 mm	100°C, 360 min	868.14±5.04^{k}	114.42±0.66^{j}
HD-0.2 mm	100°C, 360 min	823.55±15.77^{j}	118.01±2.26^{k}
Conventional extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
SLE-Ac	40°C, 360 min	302.39±2.73^{e}	6.17±0.06^{c}
SLE-Ac	R. T, 360 min	288.67±2.87^{e}	4.56±0.05b^{c}
Soxhlet-Ac	56°C, 360 min	645.49±16.45^{h}	40.54±1.03^{f}
High pressure extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
PLE-Ac	120°C, 30 min, 10 MPa	738.98±9.27^{i}	64.29±0.81^{h}
PLE-Ac-EtOH	40°C, 30 min, 10 MPa	1010.64±4.69^{m}	152.91±0.71^{l}
PLE-Ac-EtOH	120°C, 30 min, 10 MPa	1171.04±19.71^{n}	195.21±3.29^{o}
PLE-Ac-EtOH	120°C, 60 min, 10 MPa	817.49±4.63^{j}	58.37±0.33^{g}
PLE-Ac-EtOH/H_2O (1:1)	120°C, 30 min, 10 MPa	1020.28±6.51^{m}	295.78±1.89^{p}
PLE-Ac-EtOH-H_2O	120°C, 30 min, 10 MPa	737.53±3.81^{i}	164.25±0.85^{m}
PLE-Ac-Et/H_2O-H_2O	120°C, 30 min, 10 MPa	210.03±2.97^{d}	9.41±0.13^{d}

Referred average values of five determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

DPPH$^{\bullet}$ (2,2-diphenyl-1- picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. The delocalisation on the DPPH• molecule determines the occurence of a purple colour, with an absorbtion band with a maximum around 520nm. When DPPH• reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet colour. Therefore, the absorbance diminution

depends linearly on the antioxidant concentration. Trolox is used as standard antioxidant [167,168]. The ABTS and DPPH scavenging capacities of various non-polar and polar extracts are presented in **Table 20** and **Table 21**, respectively. In the ABTS scavenging assay extracts activity varied from 16.4 mg TE/g extract to 1171.0 mg TE/g extract, evaluated extracts yields and recalculated obtained values to DW, they varied from 0.25 mg TE/g DW to 295.8 mg TE/g DW. Non-polar extracts activity varied from 16.44 mg TE/g extract to 480.52 mg TE/g extract (0.25-14.80 mg TE/g DW). Among conventional extraction methods the lowest scavenging capacity was obtained in Soxhlet extracts (on average 19.91 mg TE/g extract, corresponding to 0.60 mg TE/g DW) while hexane-soluble extract obtained after SLE possessed up to 20-fold higher activity (386.11 mg TE/g extract, equal to 6.87 mg TE/g DW). Non-polar extracts obtained by high pressure extraction method – SFE-CO_2 corresponded scavenging capacity from 16.44 mg TE/g extract to 111.60 mg TE/g extract (0.25-2.23 mg TE/g DW). Almost all SFE-CO_2 extracts in all temperature range (25 -70°C), with or without 5% EtOH modifier corresponded activity on average 23.39 mg TE/g extract (0.56 mg TE/g DW) except two extracts (one isolated at 70°C, +5% EtOH and another – isolated at 70°C from plant material after hydrodistillation) whose activity were 61.13 mg TE/g extract (1.69 mg TE/g DW) and 111.60 mg TE/g extract (2.23 mg TE/g DW). Non-polar, hexane-soluble PLE extract had the highest radical scavenging capacity – 480.52 mg TE/g extract (14.80 mg TE/g DW). Polar extracts isolated by conventional extraction methods (Soxhlet extraction and SLE and hydrodistillation) antioxidant activity varied in range 288.67 mg TE/g extract (4.56 mg TE/g DW) – 940.13 mg TE/g extract (179.66 mg TE/g DW). SLE extracts obtained at different temperatures statistically not differed, while those obtained in hydrodistillation differed: from unground fraction isolated extract had the highest value among hydrodistillation extracts (940.16 mg TE/g extract) from 0.2 mm size fraction obtained extract had the lowest value among them (823.55 mg TE/g extract), 0.5 mm and 1 mm size fraction extracts did not different between each other. Polar extracts isolated by PLE, $TEAC_{ABTS}$ values decreased as follows: PLE-Ac-EtOH (T-120°C, τ-30 min) (1171 mg TE/g extract) > PLE-Ac-EtOH/H_2O (1020.3 mg TE/g extract) > PLE-Ac-EtOH (T-40°C, τ-30 min) (1010.6 mg TE/g extract) > PLE-Ac-EtOH (T-120°C, τ-60 min) (817.5 mg TE/ g extract) > PLE-Ac (739 mg TE/ g extract) > PLE-Ac-EtOH-H_2O (737.5 mg TE/ g extract) > PLE-Ac-EtOH/H_2O-H_2O (210 mg TE/g extract). Gomez-Estaca et al. (2009) investigated oregano and rosemary antioxidant properties on different gelatin films. In this study were investigated aqueous extracts (dried herbs were soaked with distilled water at 45°C). $ABTS^{\bullet+}$ method values expressed as mg of ascorbic acid equivalents/ml of extract. For oregano, it was 1.05 AAE/ml of sample, for rosemary – 0.14 AAE/ml sample [172].

In DPPH scavenging assay extracts activity varied form 6.24 mg TE/g extract to 569.87 mg TE/g extract. Polar extracts obtained both conventional extraction methods and high pressure

extraction methods corresponded statistically not different scavenging capacity from 6.24 mg TE/g extract to 10.17 mg TE/g extract on average 16.22 mg TE/g extract (0.20 mg TE/g DW).

Table 21. DPPH scavenging assay values obtained in various non-polar and polar extracts isolated by different conventional and high pressure extraction techniques, expressed as mg TE/g extract and mg TE/g DW

Extract	Extraction conditions	$TEAC_{DPPH}$	
		mg TE/g sample	*mg TE/g DW*
Non-polar extracts			
Conventional extractions (from starting plant material):			
Soxhlet-He-ungroud	69°C, 360 min	6.30±0.14^{a}	0.15±0.00^{ab}
Soxhlet-He-1 mm	69°C, 360 min	7.45±0.17^{a}	0.22±0.01^{ab}
Soxhlet-He-0.5 mm	69°C, 360 min	6.24±0.05^{a}	0.20±0.00^{ab}
Soxhlet-He-0.2 mm	69°C, 360 min	7.33±0.14^{a}	0.24±0.00^{ab}
SLE-He	50°C, 360 min	7.75±0.23^{a}	0.14±0.00^{ab}
High pressure extractions (from starting plant material):			
SFE-CO_2	25°C, 210 min, 45 MPa	9.32±0.20^{a}	0.12±0.00^{a}
SFE-CO_2	40°C, 210 min, 45 MPa	8.30±0.33^{a}	0.13±0.01^{a}
SFE-CO_2	50°C, 210 min, 45 MPa	9.91±0.24^{a}	0.20±0.00^{ab}
SFE-CO_2	70°C, 210 min, 45 MPa	9.09±0.17^{a}	0.21±0.00^{ab}
SFE-CO_2 (5% EtOH)	40°C, 210 min, 45 MPa	8.21±0.24^{a}	0.24±0.01^{ab}
SFE-CO_2 (5% EtOH)	50°C, 210 min, 45 MPa	7.58±0.19^{a}	0.24±0.01^{ab}
SFE-CO_2 (5% EtOH)	70°C, 210 min, 45 MPa	8.93±0.16^{a}	0.25±0.00^{ab}
SFE-CO_2 (after HD)	70°C, 210 min, 45 MPa	10.17±0.19^{a}	0.20±0.00^{ab}
PLE-He	50°C, 15 min, 10 MPa	6.99±0.49^{a}	0.22±0.02^{ab}
Polar extracts			
Conventional extractions (from starting plant material):			
HD-Unground	100°C, 360 min	364.13±7.26^{g}	69.59±1.39^{l}
HD-1 mm	100°C, 360 min	378.76±4.67^{i}	48.67±0.40^{h}
HD-0.5 mm	100°C, 360 min	352.51±5.45^{f}	46.46±0.72^{g}
HD-0.2 mm	100°C, 360 min	481.0±13.92^{l}	68.93±2.00^{l}
Conventional extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
SLE-Ac	40°C, 360 min	131.74±1.36^{d}	2.69±0.03^{c}
SLE-Ac	R. T, 360 min	110.69±2.44^{c}	1.75±0.04b^{c}
Soxhlet-Ac	56°C, 360 min	124.03±3.75^{d}	7.79±0.24^{d}
High pressure extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
PLE-Ac	120°C, 30 min, 10 MPa	367.21±5.39^{h}	37.95±0.47^{f}
PLE-Ac-EtOH	40°C, 30 min, 10 MPa	403.98±6.22^{j}	66.65±0.63^{k}
PLE-Ac-EtOH	120°C, 30 min, 10 MPa	569.87±6.70^{m}	61.12±0.94^{i}
PLE-Ac-EtOH	120°C, 60 min, 10 MPa	394.73±2.45^{j}	28.18±0.18^{e}
PLE-Ac-EtOH/H_2O (1:1)	120°C, 30 min, 10 MPa	440.54±4.15^{k}	127.71±1.20^{m}
PLE-Ac-EtOH-H_2O	120°C, 30 min, 10 MPa	291.01±9.73^{e}	64.81±2.17^{j}
PLE-Ac-EtOH/H_2O-H_2O	120°C, 30 min, 10 MPa	77.53±1.75^{b}	3.47±0.07^{c}

Referred average values of five determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

Polar extracts isolated by conventional extraction methods antioxidant activity values varied from 110.69 mg TE/g extract to 481.0 mg TE/g extract (1.75 mg TE/g DW – 69.59 mg TE/g DW). Soxhlet and SLE corresponded similar values, while extracts after hydrodistillation had up to 3-fold higher values. Polar extracts obtained after various PLE steps values increased as follows: PLE-Ac-EtOH/H_2O-H_2O (77.53 mg TE/g extract) < PLE-Ac-EtOH-H_2O (291.01 mg TE/ g extract) < PLE-Ac (367.21 mg TE/ g extract) < PLE-Ac-EtOH (T-120°C, τ-60 min) (394.73 mg TE/g extract) < PLE-Ac-EtOH (T-40°C, τ-30 min) (403.98 mg TE/g extract) < PLE-Ac-

EtOH/H_2O (440.54 mg TE/g extract) < PLE-Ac-EtOH (T-120°C, τ-30 min) (569.87 mg TE/g extract). Skotti et al. (2014) investigated $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ antioxidant activity in different extracts obtained from oregano (*O. vulgare*). Fresh, crushed plant material was mixed with deionized water and then were treated at three different conditions: (a) at 85°C, (b) at room temperature and (c) at room temperature with the assistance of ultrasound. Each treatment lasted for15 minutes. $ABTS^{\bullet+}$ values for a process extract was 3.4 μmol Trolox/mL, for b process 1.6 μmol Trolox/mL and for c – 1.7 μmol Trolox/mL. $DPPH^{\bullet}$ scavenging assay values for a process was 3.2 μmol Trolox/mL for b – 1.5 μmol Trolox/mL, for c – 1.6 μmol Trolox/mL [173]. Su et al. (2007) ivestigated oregano leaves acetone-soluble extracts antioxidant activithy. Oregano leaves were crushed and extracted 15 h with acetone. $ABTS^{\bullet+}$ value was 337 μmol TE/g plant, also TPC value was measured – 5.48 mg GAE/g plant [174].

3.4.3. ORAC assay in isolated non-polar and polar extracts

The ORAC (oxygen radical absorption capacity) assay: the method measures the antioxidant scavenging activity against the peroxyl radical, induced by 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH), at 37°C. Fluorescein was used as the fluorescent probe. The loss of fluorescence was an indicator of the extent of the decomposition, from its reaction with the peroxyl radical [167,168]. In the ORAC assay polar and non-polar extracts activity varied from 49.51 mg TE/g sample to 3403.12 mg TE/g sample (**Table 22**). Non-polar extracts isolated by conventional and high pressure methods ORAC values varied from 49.51 mg TE/g extract to 390.11 mg TE/g extract corresponding 1.52 mg TE/g DW – 9.17 mg TE/g DW. Almost all in these extracts obtained values statistically not differed (on average 206.44 mg TE/g extract), except the lowest value obtained in PLE-He extract, and the highest one obtained in SFE-CO_2 extract isolated from plant material after hydrodistillation. Polar extracts isolated by conventional extraction method ORAC values varied from 1082.86 mg TE/g extract to 2081.62 mg TE/g extract. SLE-Ac extracts were similar and statistically not differed, while values obtained in hydrodistillation extracts differed: extracts from unground and 0.5 mm fractions plant material corresponded on average 1154.91 mg TE/g extract and 1 mm and 0.2 mm fractions corresponded on 2065.87 mg TE/g extract. Polar extracts isolated by PLE $TEAC_{ORAC}$ values increased as follows: PLE-Ac-EtOH/H_2O-H_2O (680.04 mg TE/g extract) PLE-Ac (1579.03 mg TE/g extract) < PLE-Ac-EtOH-H_2O (1996.2 mg TE/ g sample) < PLE-Ac-EtOH/H_2O (1:1) (2021.2 mg TE/g sample) < PLE-Ac-EtOH (T-120°C, τ-60 min) (3251.4 mg TE/g sample) < PLE-Ac-EtOH (T-40°C, τ-30 min) (3394.3 mg TE/ g sample) < PLE-Ac-EtOH (T-120°C, τ-30 min) (3403.1 mg TE/ g sample). As could be seen from all antioxidant activity measurement methods for non-polar extracts almost in all cases there is no big

difference between conventional and high pressure extraction methods for antioxidant fraction isolation.

Table 22. ORAC assay values obtained in various non-polar and polar extracts isolated by different conventional and high pressure extraction techniques, expressed as mg TE/g extract and mg TE/g DW

Extract	Extraction conditions	$TEAC_{ORAC}$	
		mg TE/g sample	*mg TE/g DW*
Non-polar extracts			
Conventional extractions (from starting plant material):			
Soxhlet-He-ungroud	69°C, 360 min	190.38±16.20^{ab}	4.47±0.38^{a}
Soxhlet-He-1 mm	69°C, 360 min	124.57±12.28^{ab}	3.76±0.37^{a}
Soxhlet-He-0.5 mm	69°C, 360 min	142.88±12.78^{ab}	4.69±0.42^{a}
Soxhlet-He-0.2 mm	69°C, 360 min	158.90±12.18^{ab}	5.12±0.39^{a}
SLE-He	50°C, 360 min	104.23±4.83^{a}	1.86±0.09^{a}
High pressure extractions (from starting plant material):			
SFE-CO_2	25°C, 210 min, 45 MPa	206.75±14.96^{ab}	2.71±0.20^{a}
SFE-CO_2	40°C, 210 min, 45 MPa	198.04±18.08^{ab}	2.99±0.27^{a}
SFE-CO_2	50°C, 210 min, 45 MPa	196.97±15.97^{ab}	3.96±0.32^{a}
SFE-CO_2	70°C, 210 min, 45 MPa	218.03±19.12^{ab}	5.01±0.44^{a}
SFE-CO_2 (5% EtOH)	40°C, 210 min, 45 MPa	314.00±18.82^{ab}	9.17±0.55^{a}
SFE-CO_2 (5% EtOH)	50°C, 210 min, 45 MPa	245.86±14.95^{ab}	7.70±0.47^{a}
SFE-CO_2 (5% EtOH)	70°C, 210 min, 45 MPa	274.56±7.27^{ab}	7.58±0.20^{a}
SFE-CO_2 (after HD)	70°C, 210 min, 45 MPa	390.11±18.89^{b}	7.80±0.44^{a}
PLE-He	50°C, 15 min, 10 MPa	49.51±4.98^{a}	1.52±0.15^{a}
Polar extracts			
Conventional extractions (from starting plant material):			
HD-Unground	100°C, 360 min	2050.11±132.76^{i}	391.78±25.37^{e}
HD-1 mm	100°C, 360 min	1727.40±91.98^{g}	221.91±11.82^{d}
HD-0.5 mm	100°C, 360 min	2081.62±200.92^{i}	274.36±30.04^{d}
HD-0.2 mm	100°C, 360 min	1759.26±57.07^{gh}	252.10±8.17^{d}
Conventional extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
SLE-Ac	40°C, 360 min	1082.86±100.97^{d}	22.09±2.10^{ab}
SLE-Ac	R. T, 360 min	1226.96±130.39^{de}	19.39±2.11^{ab}
Soxhlet-Ac	56°C, 360 min	1458.36±150.47^{ef}	91.58±9.06^{bc}
High pressure extractions (from plant residues after SFE-CO_2 (50°C, 210 min, 45 MPa)):			
PLE-Ac	120°C, 30 min, 10 MPa	1579.03±141.59^{fg}	138.94±12.32^{c}
PLE-Ac-EtOH	40°C, 30 min, 10 MPa	3394.32±177.51^{j}	456.63±162.54^{e}
PLE-Ac-EtOH	120°C, 30 min, 10 MPa	3403.12±350.39^{j}	567.30±58.41^{f}
PLE-Ac-EtOH	120°C, 60 min, 10 MPa	3251.40±200.43^{j}	232.15±14.31^{d}
PLE-Ac-EtOH/H_2O (1:1)	120°C, 30 min, 10 MPa	2021.21±198.25^{hi}	585.95±57.47^{f}
PLE-Ac-EtOH-H_2O	120°C, 30 min, 10 MPa	1996.15±184.47^{hi}	444.54±41.08^{e}
PLE-Ac-EtOH/H_2O-H_2O	120°C, 30 min, 10 MPa	680.04±55.58^{c}	30.47±2.29^{ab}

Referred average values of five determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

Considering that conventional extractions took 360 min for constituents isolation, preferable would be SFE-CO_2 and PLE, because of shorter time and in some cases higher antioxidant activity of obtained extracts. For polar extracts isolation likewise PLE would be superior against conventional extraction methods, because of its short time and high antioxidant activity values of isolated constituents.

3.4.4. Antioxidant activity of starting plant materials and solid residues

There are many direct and indirect ways to measure bioactive compounds antioxidant activity. Preliminary raw material antioxidant activity measures are made applying various *in vitro* analytical methods. *In vitro* model systems phenolic compounds as potential antioxidants properties are measured relatively simple and controlled conditions of parameters. The most commonly studied material is water-soluble, other solvents extracts antioxidant activity, but using different solvents not all bioactive compounds can be extracted, and some of them could be harmed by solvents. Insoluble bioactive compounds antioxidant activity may be evaluated applying the QUENCHER procedure. The QUENCHER (QUick, Easy, New, CHEap and Reproducible) approach does not require previous extractions or hydrolysis to release bioactive compounds. The solid sample is directly brought in contact with a reagent solution containing radicals. The radicals are quenched by antioxidants due to the contact at the solid-liquid interface and thereby a decolourization occurs. Also, QUENCHER procedure does not depend on reaction mechanism and could be used with all antioxidant activity assessment tests.

TPC values in plant residues after all extractions varied in a wide range from 1.73 mg GAE/ g DW to 217.1 mg GAE/g DW (**Table 23**). In oregano plant material prior, all extractions obtained TPC value was 116.6 mg GAE/ g DW. TPC values in solid residues increased as follows: PLE-Ac-EtOH/H_2O (1:1), PLE-Ac-EtOH-H_2O, PLE-AcEtOH/H_2O-H_2O < HD-1 mm – unground, 50°C-70°C, HD SFE-CO_2, Soxhlet-Ac, SLE-Ac (R.T) (6.6-11.2-fold) < 25°C, 50°C, SLE-Ac (40°C), HD-0.2 – 0.5 mm (1.1-1.3-fold) < Soxhlet-He – 0.5 – unground, 50°C, Et40°C SFE-CO_2, SLE-He, PLE-Ac, PLE-Ac-EtOH (T-40°C, τ-30 min) PLE-Ac-EtOH (T-120°C, τ-60 min) (1.1-1.4-fold) < Soxhlet-He-0.2 mm, SLE-He, PLE-Ac-EtOH (T-120°C, τ-30 min) (1.3-1.6-fold).

$ABTS^{\bullet+}$ scavenging assay was carried out with plant material prior all extractions and with all solid residues obtained after different extractions, or different steps of extraction. Values expressed as mg TE/ g DW. As it could be seen from table if extracts distinguished low activity, solid residues remained with high activity, and vice versa. Obtained values varied from 13.2 mg TE/ g DW to 463 mg TE/ g DW. $TEAC_{ABTS}$ increases as follows: PLE-Ac-EtOH/H_2O, PLE-Ac-H_2O (T-40°C, τ-30 min), PLE-Ac-EtOH/H_2O-H_2O < HD-unground (6.4-fold) < PLE-Ac-EtOH (T-120°C, τ-60 min), SLE-He, Soxlet-He-0.2 mm, unground, Et40°C, Et70°C SFE-CO_2, Soxhlet-Ac (1.5-1.9-fold) < 25-70°C, Et50°C, 70°C HD SFE-CO_2, HD-0.2-1mm, PLE-Ac, PLE-Ac-EtOH (T-120°C, τ-30 min), oregano, Soxhlet-0.5-1mm, SLE-Ac-(R.T-40°C) (1.1-1.4-fold) < PLE-Ac-EtOH (T-120°C, τ-60 min) (1.2-fold) < PLE-He (1.2-fold).

$DPPH^{\bullet}$ scavenging assay values in plant residues had similar tendency as in ABTS assay. $TEAC_{DPPH}$ increased as follows: PLE-Ac-EtOH/H_2O, PLE-Ac-EtOH-H_2O, PLE-Ac-EtOH/H_2O-H_2O < HD-unground, 70°C HD SFE-CO_2 (1.2-3.6-fold) < 40°C, 70°C Et40-Et70°C SFE-CO_2,

HD-0.2, 1mm, oregano (1.5-2.2-fold) < PLE-EtOH (T-40°C, τ-30 min), SLE-He, Soxlet-He-0.2mm-unground, 25°C , 50°C, SFE-CO_2, Soxhlet-Ac, HD-0.5 mm, SLE-Ac-R.T-40°C, PLE-Ac, PLE-EtOH (T-120°C, τ-30 min), PLE-EtOH (T-120°C, τ-60 min), PLE-He (up to 1.9-fold).

ORAC values in plant residues increased as follows: PLE-Ac-EtOH/H_2O-H_2O, PLE-Ac-EtOH/H_2O (1:1) < PLE-Ac-EtOH-H_2O (3.5-fold) < 25°C – 40°C, Et40°C, Et70°C SFE-CO_2 (5.6-6.5-fold) < PLE-Ac, PLE-Ac-EtOH (T-40°C, τ-30 min), PLE-Ac-EtOH (T-120°C, τ-60 min), 50°C-70°C, Et50°C, HD SFE-CO_2, SLE-Ac (R.T), HD-0.2-1 mm, Soxhlet-He-ungorund, oregano (1.2-1.6-fold), < Soxhlet-He-0.2-1 mm, PLE-He, SLE-He, SLE-Ac (40°C), Soxhlet-Ac (1.1-1.4-fold).

As could be seen from obtained results, high-pressure fractionation scheme reduced the initial total phenolic content and antiradical capacity of starting plant material before SFE-CO_2 by 94-98%, showing its efficiency to remove the major portion of antioxidatively active constituents from oregano. Using conventional extraction methods for antioxidant fraction isolation is way more less effective than using high pressure extractions methods. Antioxidant activity could be very similar, but keeping in mind that conventional extractions takes much longer (to achieve higher extraction yields) high pressure extractions are way more economic.

Table 23. Antioxidant activity in solid residues after conventional and high pressure extractions (each step) and plant material prior extraction, extracted as mg GAE/g DW or mg TE/g DW

Sample	Folin-Ciocalteu's	ABTS	DPPH	ORAC
	mg GAE/g DW	*mg TE/g DW*	*mg TE/g DW*	*mg TE/g DW*
Oregano	116.56±11.47^{jklm}	403.68±23.50^{fghijkl}	162.44±14.65^{def}	529.23±56.38^{ij}
Soxhlet-He-ungroud	138.55±6.70^{n}	331.39±12.01^{cdef}	245.32±10.63^{ijkl}	538.84±35.57^{ijk}
Soxhlet-He-1 mm	107.03±4.04^{ijkl}	415.23±44.03^{ghijkl}	197.72±17.94^{gh}	727.29±41.46^{n}
Soxhlet-He-0.5 mm	123.16±11.52^{lmn}	462.99±48.95^{l}	177.55±7.49^{fgh}	763.65±62.60^{n}
Soxhlet-He-0.2 mm	217.11±18.29^{p}	307.70±20.14^{cde}	189.63±10.43^{fgh}	614.20±65.71^{klm}
SLE-He	113.66±4.62^{jklm}	276.36±18.53^{cd}	314.80±25.40^{n}	708.29±66.22^{mn}
SFE-CO_2	98.39±7.63^{ghij}	443.09±39.97^{ijkl}	275.61±7.52^{lm}	349.22±32.31^{cde}
SFE-CO_2	83.47±7.59^{defgh}	403.47±40.34^{fghijkl}	174.84±7.69^{efg}	295.11±16.50^{c}
SFE-CO_2	111.27±7.95^{mn}	363.26±10.92^{efghi}	237.24±14.99^{ij}	531.00±43.64^{ij}
SFE-CO_2	80.85±5.08^{cdefg}	368.28±19.01^{efghi}	119.77±4.46^{c}	397.02±35.72^{def}
SFE-CO_2 (5% EtOH)	112.15±8.22^{jklm}	335.48±28.94^{cdef}	128.83±8.33^{cd}	324.82±26.17^{cd}
SFE-CO_2 (5% EtOH)	71.76±6.38^{cdef}	392.99±22.87^{fghijkl}	118.69±3.56^{c}	544.06±19.20^{ijk}
SFE-CO_2 (5% EtOH)	62.67±5.70^{bc}	348.88±34.49^{defgh}	122.70±6.08^{c}	298.17±9.74^{c}
SFE-CO_2 (after HD)	56.47±4.92^{cd}	385.28±33.38^{kl}	67.18±2.36^{b}	339.61±37.03^{def}
SLE-Ac	87.95±8.52^{fghi}	420.16±38.25^{hijkl}	266.57±23.42^{klm}	478.73±34.78^{ghi}
SLE-Ac	69.63±5.56^{cde}	390.48±31.86^{fghijkl}	264.55±20.15^{jklm}	610.97±62.73^{kl}
PLE-He	182.67±5.02^{o}	646.44±45.39^{n}	299.38±13.72^{mn}	694.60±43.83^{lmn}
Soxhlet-Ac	60.11±3.05^{cd}	329.06±32.80^{defgh}	243.41±20.60^{jkl}	561.27±44.86^{jk}
HD-unground	36.91±3.47^{b}	149.25±6.59^{b}	52.07±3.50^{b}	131.12±9.75^{b}
HD-1 mm	67.31±2.89^{cdef}	329.49±32.39^{efghij}	110.90±9.54^{cd}	371.50±20.56^{efgh}
HD-0.5 mm	77.74±3.27^{efgh}	396.27±43.28^{jkl}	189.23±14.23^{hi}	436.61±18.90^{ghi}
HD-0.2 mm	78.09±3.09^{efghi}	369.32±35.61^{ghijkl}	124.90±7.26^{cde}	479.22±46.14^{ijk}
PLE-Ac	119.86±7.69^{mn}	350.95±27.93^{efghijk}	236.05±18.32^{jkl}	458.80±45.60^{hi}
PLE-Ac-EtOH	89.65±7.06^{klm}	307.50±28.07^{fghijkl}	195.39±180.79^{ijk}	315.15±19.65^{efg}
PLE-Ac-EtOH	145.44±9.34^{o}	426.02±35.86^{m}	205.84±19.76^{lm}	105.45±10.15^{b}
PLE-Ac-EtOH	85.08±6.97^{hijk}	220.72±20.18^{c}	221.44±20.07^{jkl}	398.41±35.91^{fghi}
PLE-Ac-EtOH/H_2O (1:1)	1.73±0.13^{a}	14.16±0.95^{a}	12.78±1.10^{a}	4.49±.0.35^{a}
PLE-Ac-EtOH-H_2O	3.59±0.11^{a}	13.17±1.09^{a}	10.41±0.97^{a}	13.31±1.24^{a}
PLE-Ac-Et/H_2O (1:1)-H_2O	2.22±0.08^{a}	16.22±1.40^{a}	12.13±0.75^{a}	2.63±0.16^{a}

Referred average values of five determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

Looking at Pearson correlation coefficients (**Table 24**) between different antioxidant activity indices of non-polar (Soxhlet-He, SLE-He, SFE-CO_2, SFE-CO_2/EtOH, PLE-He) and polar (hydrodistillation, Soxhlet-Ac, SLE-Ac, PLE-Ac, PLE-Ac-EtOH, PLE-Ac-EtOH/H_2O, PLE-Ac-EtOH-H_2O, PLE-Ac-EtOH/H_2O- H_2O) extracts (0.9191-0.9812 with $p < 0.05$), strong significant positive correlation between TPC and *in vitro* antiradical capacity indicators (decreasing in the following order: ABTS > DPPH > ORAC) was obtained. Lower (0.8188-0.8821 with p <0.05) Pearson coefficient values were calculated, adding unextraced oregano and solid residues' antioxidant activity results, expressed as mg GAE or TE/ g DW of starting plant material, however the same trend for correlation between TPC and other assays was obtained. These results suggest that mainly phenolic compounds are responsible for the measured *in vitro* radical scavenging properties of oregano extracts.

Table 24. Analysis of correlation between different antioxidant activity indices of non-polar and polar extracts, starting plant material and solid residues, obtained from *O. vulgare* after hydrodistillation, Soxhlet, SLE, SFE-CO_2 and PLE at various extraction conditions

Antioxidant activity indices[1]	**Pearson correlation coefficients[2]**			
	TPC	DPPH	ABTS	ORAC
Non-polar + polar extracts:				
TPC	1	0.9286****	0.9390****	0.9191****
DPPH		1	0.9812****	0.9415****
ABTS			1	0.9744****
ORAC				1
Starting plant material + solid residues after extraction:				
TPC	1	0.6754****	0.6812****	0.6441***
DPPH		1	0.6668****	0.7065****
ABTS			1	0.6911****
ORAC				1
Non-polar and polar extracts + starting plant material and solid residues:				
TPC	1	0.8554****	0.8700****	0.8195****
DPPH		1	0.8821****	0.8188****
ABTS			1	0.8415****
ORAC				1

PLE: pressurized liquid extraction; SFE-CO_2: supercritical carbon dioxide extraction; [1] expressed as mg GAE/g DW of starting plant material or mg TE/g DW of starting plant material; [2] Pearson correlation coefficients were calculated using GraphPad Prism 6.01 software (2012); *: correlation is significant at the P<0.05 level (two-tailed).

3.4.5. Rapeseed oil stability in Oxipres method

Possibilities of the selected oregano extracts to increase the oxidative stability of rapeseed oil was additionally evaluated by measuring rapeseed oil stability under the accelerated oxidation conditions (Oxipres method). For these purposes, mixtures of not refined rapeseed oil and different amount of extracts (1%, 2.5% and 5%) were prepared (**Table 25**). Oxipres method is based on accelerated oxidation measurements. Measurements lasts until secondary oxidation products are observed.

Table. 25 Rapeseed oil induction periods with various amounts of different samples

Sample	IP, h	Sample	IP, h
Control	3.88±0.11	SFE-CO_2 5%	3.35±0.18
SFE-CO_2 1%	4.29±0.17	PLE-Ac 1%	6.46±0.10
SFE-CO_2 2.5%	3.44±0.08	PLE-EtOH 1%	4.27±0.40

Referred average values of duplicate ± SD.

As it could be clearly seen from obtained results (**Fig. 22**), SFE-CO_2 extracts show antioxidant properties and delay oil oxidation only to a certain concentration. The best effect was achieved with 1% of extract in rapeseed oil, extending its induction period by 11%. However, higher amounts of SFE-CO_2 (2.5% and 5%) extracts worked like prooxidants and reduced induction period in comparison with control for 13% and 16%, respectively. As could be seen from total phenolic content measurements, oregano extract possesses high values, but also it is reported that phenolic compounds at small concentration could work as antioxidants, but at higher concentrations (especially flavonoids) could act as prooxidants [175]. Prooxidants may induce reactive radicals or inhibit antioxidant systems. Both, PLE-Ac and PLE-EtOH extracts worked like antioxidants and extended induction period. Rape seed oil with PLE-Ac extract in it induction period was 67% longer than pure rape seed oil. With PLE-Ac-EtOH extract induction period was longer 10% than control sample.

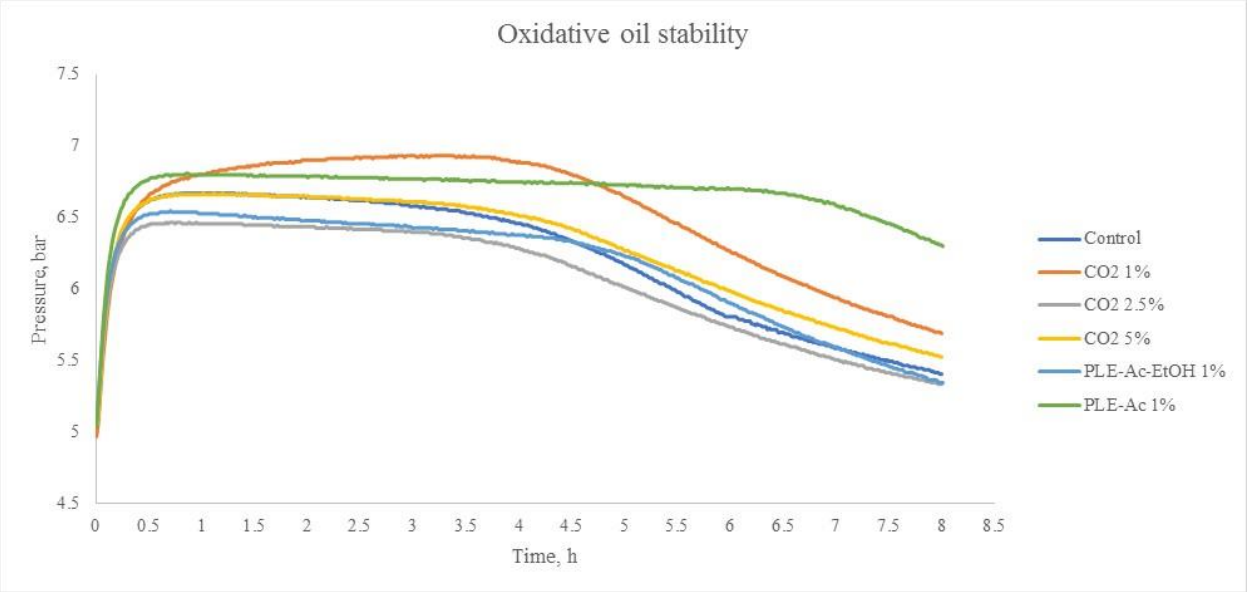

Fig. 22 Oxidative oil stability, measured by oxipres apparatus

Trojakova and coworkers (2000) conducted a study with sage and rosemary extracts in refined rapeseed and sunflower oil. Extracts were prepared by soaking plant materials in hexane, acetone and ethanol for 24 h in room temperature and ambient pressure in the dark. In all oils, there were used 0.05% of different extracts and oxipres carried out at 100°C. With sage acetone, ethanol, hexane extracts induction periods in rapeseed oil were 11.26h, 11.26h, 11.70h respectively (control sample – 8.80h). In sunflower oil – 8.3h, 8.03h, 8.77h, respectively (control sample – 6.64h). With same solvents rosemary extracts in rapeseed oil induction periods were as follows: with acetone -

12.50h, with ethanol – 12.06h. In sunflower oil: with acetone – 9.56h, with ethanol – 8.63h [176]. Comparing these authors results with results obtained in this research it is clearly seen that, their oxidation period took much more longer. It could be different reason to explain this tendency. First of all, in this study process was carried out in higher temperature (110°C) and different extract concentration may have some influence.

3.5. Phytochemical characterization of non-polar and polar oregano extracts

3.5.1. UPLC-QTOF-MS analysis of the selected extracts

The preliminary phytochemical characterization of selected extracts (non-polar: Soxhlet (unground, 1 mm, 0.5 mm, 0.2 mm, T-69°C, τ-360 min), SLE-He (T-50°C, τ-360 min), SFE-CO_2 at 50°C, SFE-CO_2 at 50°C + EtOH (τ-210 min, P-45 MPa), PLE-He (T-50°C, τ-15 min, P-10 MPa), polar: hydrodistillation (unground, 1 mm, 0.5 mm, 0.2 mm, T-100°C, τ-240 min), SLE-Ac (T-40°C, τ-360 min), PLE-Ac (T-120, τ-30 min, P-10 MPa), PLE-EtOH (T-120, τ-30 min, P-10 MPa), PLE-EtOH/H_2O (T-120, τ-30 min, P-10 MPa), PLE-H_2O (T-120, τ-30 min, P-10 MPa), PLE-H_2O (T-120, τ-30 min, P-10 MPa)) was analyzed by UPLC-QTOF-MS. Retention times, accurate mases, molecular ion [M-H] formulas and presence of compounds (+) in extracts are presented in **Tables 26** (for non-polar extracts) and **27** (polar extracts). Compounds presence were identified using database (ChemSpider). Concerning the variability of the data the relative standard deviations of peak areas were < 5%. In non-polar extracts were identified 16 constituents. In **Table 26** compound **1** (197.0455 m/z) correlated to molecular ion [M-H] formula $C_9H_9O_5$ and using database was identified as syringic acid. Compounds **2** (153.0193 m/z, $C_7H_5O_4$) **3 (**137.0244 m/z, C7H5O_3), **4 (**179.035 m/z, $C_9H_7O_4$), **5 (**121.0295 m/z, $C_7H_5O_2$), **6** (225.0768 m/z, $C_{11}H_{13}O_5$) also were identified as acids with corresponding names as gentisic acid, hydroxybenzoic acid, caffeic acid, benzoic acid, dihydroxisinapic acid. Compound **7** (343.0823 m/z) correlated to molecular ion [M-H] formula $C_{18}H_{15}O_7$ was identified as flavone glucoside. At 4.7-4.8 min eluting compounds **8** (305.1758 m/z, $C_{18}H_{24}O_4$) and **9** (293.1758 m/z, $C_{17}H_{15}O_7$) corresponded to two structural similar compounds known as capsiate and capsaicin derivative. Both, **10** and **11** compounds (295.2279 m/z, $C_{18}H_{31}O_3$; 297.2435 m/z, $C_{18}H_{33}O_3$) were identified as hydroxyoctadecadienoic acids, because from this preliminary analysis not possible to surely know where the double bonds or side chain substituents are presented. Compound **12** (205.1598 m/z) correlated to molecular ion [M-H] formula $C_{14}H_{21}O$ was identified as octylphenol. Compounds **13** (455.3531 m/z, $C_{30}H_{47}O_3$), **14** (277.2173 m/z, $C_{18}H_{29}O_2$), **15** (279.233 m/z, $C_{18}H_{31}O_2$), **16** (255.233 m/z, $C_{16}H_{31}O_2$) corresponded well know fatty acids such as oleanolic acid, linolenic acid, linoleic acid and palmitic acid.

Table 26. Non-polar constituents preliminary phytochemical composition by UPLC-QTOF-MS

Compound	UPLC-QTOF-MS			Conventional extractions						High pressure extractions	
				Soxhlet-He				*SLE*	*SFE-CO_2*		*PLE*
	RT (min)	*MS [M-H]$^-$ m/z*	*Formula [M-H]*	*Unground*	*1 mm*	*0.5 mm*	*0.2 mm*	*50°C*	*50°C (+EtOH)*	*50°C*	*50°C*
Syringic acid	1.3-1.4	197.0455	$C_9H_9O_5$			+					
Gentisic acid	1.4	153.0193	$C_7H_5O_4$						+		
Salycilic acid	1.7	137.0244	$C_7H_5O_3$						+	+	
Caffeic acid	1.9-2.0	179.035	$C_9H_7O_4$					+	+		
Benzoic acid	2.0-2.1	121.0295	$C_7H_5O_2$	+					+	+	
Dihydroxisinapic acid	2.2	225.0768	$C_{11}H_{13}O_5$						+	+	
Flavone glucoside	3.1-3.2	343.0823	$C_{18}H_{15}O_7$	+							
Capsiate	4.7-4.8	305.1758	$C_{18}H_{25}O_4$	+	+	+	+	+	+	+	+
Capsaicin derivative	4.8	293.1758	$C_{17}H_{25}O_4$	+	+		+	+	+	+	+
Hydroxyoctadecadienoic acid	6.1-6.2	295.2279	$C_{18}H_{31}O_3$	+	+	+	+	+	+	+	+
Hydroxyoctadecadienoic acid	6.4-6.5	297.2435	$C_{18}H_{33}O_3$	+	+	+	+	+	+	+	+
Octylphenol	6.7-6.8	205.1598	$C_{14}H_{21}O$	+	+	+	+	+	+	+	+
Oleanolic acid	7.4-7.5	455.3531	$C_{30}H_{47}O_3$	+	+	+	+	+	+	+	+
Linolenic acid	7.6	277.2173	$C_{18}H_{29}O_2$	+	+	+	+	+	+	+	+
Linoleic acid	8.1-8.2	279.233	$C_{18}H_{31}O_2$	+	+	+	+	+	+	+	+
Palmitic acid	8.6-8.7	255.233	$C_{16}H_{31}O_2$	+	+	+	+	+	+	+	+

Table 27. Polar constituents preliminary phytochemical composition by UPLC-QTOF-MS

Compound	*UPLC-QTOF-MS*			**Conventional extractions**					**High pressure extractions**				
				Hydrodistillation				*SLE-Ac*	*PLE*				
	RT (min)	*MS [M-H]⁻ m/z*	*Formula [M-H]*	*Unground*	*1 mm*	*0.5 mm*	*0.2 mm*	*40°C*	*Ac*	*EtOH*	*EtOH/H_2O*	*H_2O*	*H_2O*
Glucose	0.3-0.4	179.0561	$C_6H_{11}O_6$	+	+	+	+	+	+	+	+	+	+
Gluconic acid	0.3-0.4	195.051	$C_6H_{11}O_7$	+	+	+	+			+	+	+	
Quinic acid	0.3-0.5	191.056	$C_7H_{11}O_6$	+	+	+	+		+	+	+	+	+
Malic acid	0.3-0.5	133.0142	$C_4H_5O_5$	+	+	+	+				+	+	+
Hydroxyheptanoic acid	0.4-0.5	387.1144	$C_{13}H_{23}O_{13}$	+	+	+	+	+	+	+	+	+	+
Celobiose	0.4-0.5	683.2251	$C_{24}H_{43}O_{22}$	+	+	+	+	+	+	+	+		
Glucaric acid	0.4	209.0303	$C_6H_9O_8$	+	+	+	+		+	+	+	+	+
Sucrose	0.4-0.5	341.1089	$C_{12}H_{21}O_{11}$	+	+	+	+	+	+	+	+	+	+
Citric acid	0.9	191.0197	$C_6H_7O_7$	+	+	+	+			+	+	+	+
Protocatechuic acid glucoside	1.1	315.0722	$C_{13}H_{15}O_9$	+	+	+	+	+	+	+	+	+	+
Syringic acid	1.3-1.4	197.0455	$C_9H_9O_5$	+	+	+	+	+	+	+	+	+	+
Gentisic acid	1.4	153.0193	$C_7H_5O_4$	+	+	+	+	+	+	+	+	+	+
Salycilic acid	1.7	137.0244	$C_7H_5O_3$	+	+	+	+	+	+	+	+	+	+
Caffeic acid	1.9-2.0	179.035	$C_9H_7O_4$	+	+	+	+	+	+	+	+	+	+
Dihydroxisinapic acid	2.2	225.0768	$C_{11}H_{13}O_5$	+	+	+	+	+	+	+	+	+	+
Rutin	2.2-2.3	609.1461	$C_{27}H_{29}O_{16}$	+	+			+	+	+	+	+	+
Scutellarin	2.4-2.5	461.0725	$C_{21}H_{17}O_{12}$	+	+	+	+		+	+	+	+	+
Coumarin glucoside	2.7	323.0772	$C_{15}H_{15}O_8$	+	+	+	+	+	+	+	+	+	
Rosmarinic acid	2.8-2.9	359.0772	$C_{18}H_{15}O_8$							+			
Flavone glucoside	3.1-3.2	343.0823	$C_{18}H_{15}O_7$	+	+	+	+	+	+	+	+	+	
Capsaicin derivative	4.8	293.1758	$C_{17}H_{25}O_4$	+	+	+	+	+	+	+	+	+	+
Hydroxyoctadecadienoic acid	6.1-6.2	295.2279	$C_{18}H_{31}O_3$					+	+		+		+
Hydroxyoctadecadienoic acid	6.4-6.5	297.2435	$C_{18}H_{33}O_3$	+	+	+	+	+	+	+	+	+	
Octylphenol	6.7-6.8	205.1598	$C_{14}H_{21}O$	+	+	+	+	+	+	+	+	+	
Oleanolic acid	7.4-7.5	455.3531	$C_{30}H_{47}O_3$	+				+	+				
Linolenic acid	7.6	277.2173	$C_{18}H_{29}O_2$	+	+	+		+	+	+	+	+	
Linoleic acid	8.1-8.2	279.233	$C_{18}H_{31}O_2$	+	+			+	+	+		+	
Palmitic acid	8.6-8.7	255.233	$C_{16}H_{31}O_2$	+	+	+	+	+	+	+	+	+	+

Some of identified compounds were found only in one or several extracts, other in all of them. Compound **1** was obtained only in Soxhlet hexane-soluble extract from 0.5 mm particle size plant material, compound **2** was reported in extract obtained after SFE-CO2 with 5% EtOH modifier, compound **3** was obtained in both SFE-CO2 extracts (with and without EtOH modifier), compound **4** was reported in SLE-He and SFE-CO2 (+5% EtOH) extracts, compound 5 was obtained in Soxhlet-He with unground fraction extract and both SFE-CO2 extracts (with and without EtOH modifier), compound **6** was obtained in both SFE-CO2 extracts (with and without EtOH modifier), compound **7** was obtained in Soxhlet-He with unground fraction extract. Compounds **8-16** were reported in all investigated non-polar extracts.

Polar extracts preliminary phytochemical composition is presented in **Table 27**. Compound **1** (179.0561 m/z) correlated to molecular ion [M-H] formula $C_6H_{11}O_6$ and using database was identified as sugar derivative – glucose. Compound **2** (195.051 m/z) correlated to molecular ion [M-H] formula $C_6H_{11}O_7$ was reported as gluconic acid. At the eluting time 0.3-0.5 min were reported **3** and **4** compounds (191.056 m/z, $C_7H_{11}O_6$; 133.0142 m/z, $C_4H_5O_5$) with known names as quinic and malic acids. Compound **5** (387.1144 m/z) correlated to molecular ion [M-H] formula $C_{13}H_{23}O_{13}$ was reported as hydroxiheptanoic acid. Compound **6** (683.2251 m/z) correlated to molecular ion [M-H] formula $C_{24}H_{43}O_{22}$ was reported as celobiose. Compound **7** (209.0303 m/z) correlated to molecular ion [M-H] formula $C_6H_9O_8$ was reported as glucaric acid. Compound **8** (341.1089 m/z) correlated to molecular ion [M-H] formula $C_{12}H_{21}O_{11}$ was reported as sucrose. Compound **9** (191.0197 m/z) correlated to molecular ion [M-H] formula $C_6H_7O_7$ was reported as citric acid. Compound **10** (315.0722 m/z) correlated to molecular ion [M-H] formula $C_{13}H_{15}O_9$ was reported as protocatechic acid. Compounds **11**, **12**, **13**, **14** and **15** in polar extracts were identified the same as compounds **1**, **2**, **3**, **4**, **6** in non-polar extracts. Compound **16** (609.1461 m/z) correlated to molecular ion [M-H] formula $C_{27}H_{29}O_{16}$ was reported as rutin. Compound **17** (461.0725 m/z) correlated to molecular ion [M-H] formula $C_{21}H_{17}O_{12}$ was reported as scutellarin. Compound **18** (323.0772 m/z) correlated to molecular ion [M-H] formula $C_{15}H_{15}O_8$ was reported as coumarin glucoside. Compound **19** (359.0772 m/z) correlated to molecular ion [M-H] formula $C_{18}H_{15}O_8$ was reported as rosmarinic acid. Compound **20** (343.0823 m/z) correlated to molecular ion [M-H] formula $C_{18}H_{15}O_7$ was reported as flavone glucoside. Compounds **21**, **22**, **23**, **24**, **25**, **26**, **27** and **28** in polar extracts were identified the same as compounds **9**, **10**, **11**, **12**, **13**, **14**, **15**, **16** in non-polar extracts Non-polar extracts did not have high total phenolic content and antioxidant activity comparing them with polar extracts, because of the absence of phenolic compounds as we could see from tables. As could be seen polar extracts were identified well-known phenolic compounds with high antioxidant activity properties (such as quinic acid, caffeic acid). Large number of recorded peaks on the chromatograms indicate that the extracts are complex mixtures

of compounds, however, exact mass data obtained by UPLC-QTOF-MS was not sufficient for their identification, because mass spectra libraries give too many candidate structures for the measured masses. Purification of compounds and analysis by other spectra methods would be necessary for the more accurate identification of oregano constituents. Rosmarinic acid (acid ester of caffeic acid) is well known to have a number potentially beneficial biological effects: anti-inflammory, antioxidant. Also, rosmarinic acid is known to improve insulin sensitivity and lower plasma lipid levels, suggesting as it has antidiabetic effect. Adamako-Bonso et al. (2017), demonstrated that rosmarinic and caffeic acids posseses good antioxidant activity: rosmarinic acid DPPH radical scavenging EC_{50}=0.23 (nM), caffeic acid DPPH radical scavenging EC_{50}=0.24 (nM) [177]. In previous studies [172] oregano aqueous extracts were identified gallic acid (75.8 μg/ml extract), protocatechuic acid (74.4 μg/ml extract), caffeicacid (9.54 μg/ml extract), rosmarinic acid (177 μg/ml extract). In other studies such phenolic acid like gallic, chlorogenic, caffeic, vanillic, syringic, ferulic p-coumaric were identified, also there waere found such flavone derivatives like apigenin and luteolin. Hernandez et al (2009) in different ways extracted constituents from oregano (maceration with ethanol for 10 hours and reflux with chloroform for 2 hours). In fresh oregano chloroform extract were identified 0.0000518 mg/mL rosmarinic acid, 0.000275 mg/mL extract carnosol, 0.000125 mg/mL extract carnoisic acid, 0.0228 total phenols mg/mL extract. In dried oregano chloroform extract 0.0017 mg/mL extract rosmarinic acid, 0.0002 mg/mL extract carnosol, 0.000725 mg/mL extract carnoisic acid, 0.0393 total phenols mg/mL extract. In fresh oregano ethanol extract 0.0195 mg/mL extract rosmarinic acid, 0.000175 mg/mL extract carnosol, 0.000325 mg/mL extract carnoisic acid, 0.1032 total phenols mg/mL extract. In dried oregano ethanol extract 0.0451 mg/mL extract rosmarinic acid, 0.00025 mg/mL extract carnosol, 0.000275 mg/mL extract carnoisic acid, 0.2223 total phenols mg/mL extract [178].

3.5.2. Fatty acid determination of the selected extracts by GC-FID

Fatty acid composition is important for oregano plant material nutritional value. In different non-polar and polar extracts (Soxhlet-He, SFE-CO_2, PLE-HE, SLE-Ac, PLE-Ac) were identified 13 fatty acids, which amount was higher than 1 % (**Table 28**). Also in some extracts were found constituents, probably fatty acids which could not be identified by standard fatty acids retention indices. Identified were these fatty acids: myristic (14:0), palmitic (16:0), palmitoleic (16:1), cis-10-heptadecenoic (17:1), stearic (18:0), oleic (18:1), linoleic (18:2), arachidic (20:0), heneicosanoic (21:0), cis-11,14-eicosadienoic (20:2), behenic (21:0), cis-11,14,17-eicosatrienoic (20:3), lignoceric (24:0). The main fatty acids were heneicosanoic (amount in different extracts varied 22.80%-38.04%), palmitic acid (10.98% -21.42%), linoleic acid (9.73%-11.48%). Heneicosanoic and linoleic acid amounts varied the most in different extracts. The highest amount

of heneicosanoic acid were get in extractions carried out with acetone (in SLE – 38.81%, in PLE – 38.04%). In extractions with hexane there was obtained smaller amount, but in comparison with Soxhlet extraction there was obtained 33.46 % of heneicosanoic acid, and in PLE only 22.80%, that is almost 1.5-fold times less than in Soxhlet extraction. The lowest amount of this acid was obtained in SFE-CO_2 extraction – 19.97%. In case with palmitic acid trends remain the same: the highest amounts of this acid obtained in extractions with acetone (PLE-21.42%, SLE-20.28%). The less amount and almost the same amount obtained in extractions with hexane (PLE-14.62%, Soxhlet – 14.38), and the lowest amount obtained after SFE-CO_2 extraction – 10.98%. Linoleic acid amounts in different extracts varied not much and in all was quite similar (PLE-He – 9.82%, solid-liquid – 9.93%, SFE-CO_2 – 9.73%, Soxhlet – 11.48%, PLE-Ac – 10.45%).

Table 28. Fatty acids composition in different extracts, expressed as % of total GC area

Fatty acid	Area, %				
	Soxhlet-He	*SFE-CO_2*	*PLE-He*	*SLE-Ac*	*PLE-Ac*
Myristic	1.06±0.05	-	-	1.13±0.12	-
Palmitic	14.38±0.15^b	10.98±0.11^a	14.62±0.54^b	20.28±0.83^c	21.42±0.34^c
Palmitoleic	-	1.12±0.12	-	-	1.67±0.05
Cis-10-Heptadecenoic	-	2.28±0.14	-	-	-
Stearic	1.98±0.04^a	1.45±0.11^a	1.84±0.03^a	2.43±0.05^b	2.82±0.06^b
Oleic	3.84±0.48^a	4.32±0.48^b	3.50±0.12^a	3.46±0.08^a	3.46±0.09^a
Linoleic	11.48±0.17^b	9.73±0.62^a	9.82±0.01^a	9.93±0.24^a	10.45±0.01^a
Arachidic	1.25±0.01^a	1.17±0.06^a	1.19±0.03^a	-	-
Heneicosanoic	33.46±0.34^c	19.97±1.78^a	22.80±0.07^b	38.81±0.85^d	38.04±0.36^d
Cis-11,14-Eicosadienoic	-	1.25±0.01	1.37±0.03	-	-
Behenic	1.45±0.01	-	-	-	-
Cis-11,14,17-Eicosatrienoic	1.28±0.01^a	1.28±0.13^a	2.18±0.09^b	-	-
Lignoceric	2.25±0.13^b	1.39±0.10^a	1.47±0.15^a	-	1.19±0.07^a

Referred average values of triplicate ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

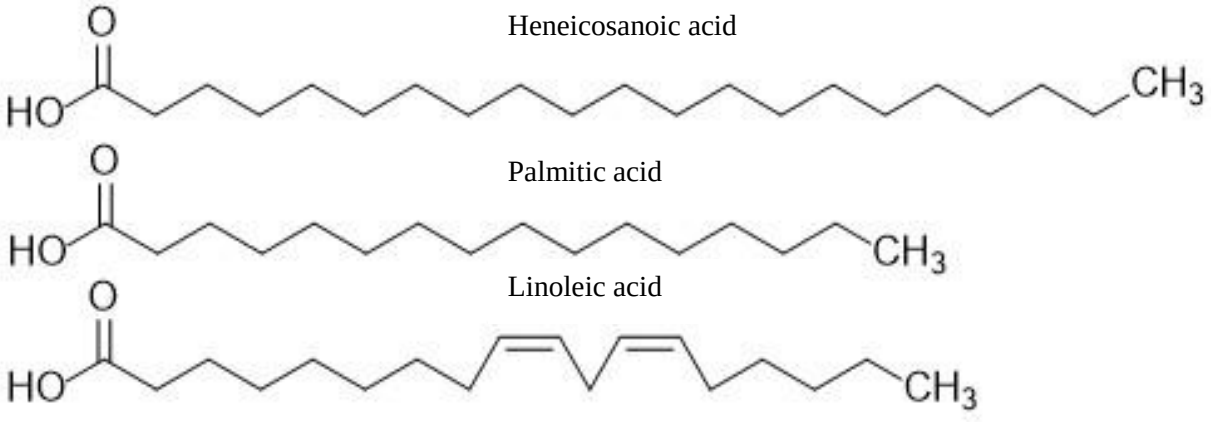

Fig. 23 Main fatty acids obtained in oregano extracts

Fatty acids composition of sage (same plant family as oregano) was investigated by Bettaieb et al (2009). These authors carried out study to identify how changes fatty acids composition under water deficit for plants. In this research were identified these fatty acids: myristic (~4%), palmitic

(~20%), palmitoleic (~3%), stearic (~3%), oleic (~15%), linoleic (~11%), linolenic (~36%), arachidic (~3%) eicosenoic (~15%). Severe water deficit for sage plant resulted a huge loss of fatty acids, some of them even was not obtained (such as palmitoleic, stearic, arachidic), but in case of eicosenoic acid during severe water deficit, it amount was up to 4-fold higher [179]. Comparing these authors results, with those obtained in this study were identified the same fatty acids such as myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidic.

3.5.3. Soxhlet extraction and SFE-CO_2 non-polar extracts GC-MS analysis

After Soxhlet and SFE-CO_2 isolated constituents were completely different looking in comparison with those obtained after hydrodistillation, so it was decided to describe their composition separately from essential oils. Obtained extracts were brownish-greenish color (while essential oils were light yellow), had typical aroma for oregano, but not as so strong as EO's, it is because during these extractions also dissolves non-volatile lipophilic constituents such as waxes, carotenoids, chlorophylls and others. Total yields of these obtained extracts were discussed earlier. The composition of constituents obtained is Soxhlet extraction and SFE-CO_2 are presented in tables 27 for Soxhlet volatile compounds and 28 for SFE-CO_2 volatile compounds. In tables are presented calculated Kovats indices, Kovats indices from literature (some compounds Kovats indices are not available in literature) and constituents in different samples (quantities expressed as percentage of the total GC peak area). The main identified compounds in extracts obtained after Soxhlet extraction were n-tetracosanol-1 (12.45-18.30%), hexacosane (5.58-9.42%), α-thujene (1.09-9.19%), α-pinene (1.03-8.01%), squalene (4.70-7.06%). In SFE-CO_2 the main obtained constituents were 1-hexacosene (10.77-42.14%), 1-docosane (18.81-33.48%), docosane 11-butyl (8.74-10.71%), sulfurous acid butyl dodecyl ester (4.46-7.64%). Kawase et al. (2013) carried out study to identify how changes oregano essential oil composition with applied different extraction methods. To dried plant material was used these extractions: Soxhlet extraction (solvents - ethanol and hexane, 4-6 hours), supercritical fluid extraction with CO_2 (120 bar, 50°C, 2 hours). In these obtained extracts were identified 14 compounds: α-thujene, sabinene, β-myrcene, p-cymene, trans-β-ocymene, γ–terpinen, trans-sabinen hydrate, cis-sabinene hydrate, 4-terpineol, α-terpineol, geraniol, thymol, carvacrol, spathulenol. In all extracts the main compound was cis-sabinene hydrate (17.4-32.6% w/w). High amount of γ–terpinen was identified in extract obtained after SFE-CO_2 (9.8% w/w), while in Soxhlet extracts this compound was not identified. Thymol amount in Soxhlet extracts varried from 17.8% w/w to 23.2% w/w, in SFE-CO_2 it was not identified.

Table 29. Compounds identified by GC-MS in hexane-soluble extracts after Soxhlet extraction

Compound	KI*	KI**	0.2 mm	0.5 mm	1 mm	Unground
m-Xylene	888	867	1.72±0.10^{c}	0.92±0.03^{b}	0.10±0.01^{a}	-
α-Thujene	919	930	6.04±0.43^{b}	9.19±0.89^{c}	5.55±0.12^{b}	1.03±0.34^{a}
α-Pinene	939	939	4.85±0.32^{b}	8.01±0.43^{c}	4.69±0.04^{b}	1.03±0.01^{a}
Myrcene	984	990	2.60±0.16^{b}	2.72±0.13^{b}	1.57±0.00^{a}	1.19±0.15^{a}
Sabinene	989	975	0.44±0.02^{b}	2.59±0.18^{c}	2.39±0.32^{c}	0.15±0.01^{a}
2-Pentanone	994	990	2.70±0.12^{a}	4.15±0.24^{c}	3.32±0.16^{b}	-
1,2,3-trimethylbenzene	1014	996	2.60±0.16^{a}	4.96±0.31^{c}	3.66±0.29^{b}	-
Limonene	1045	1029	0.82±0.04^{a}	1.55±0.09^{c}	1.18±0.09^{b}	-
E-β-Ocimene	1048	1050	0.38±0.02^{a}	1.06±0.04^{b}	0.25±0.05^{a}	-
Dopamine	1117	n.a	6.89±1.21^{d}	2.92±0.22^{c}	1.42±0.10^{a}	2.21±0.27^{b}
α-Terpineol	1205	1188	1.81±0.01^{a}	2.25±0.05^{b}	1.44±0.16^{a}	2.02±0.32^{b}
(-)-á-Bourbonene	1411	1388	0.65±0.03^{a}	1.44±0.04^{c}	1.14±0.00^{b}	1.16±0.09^{b}
Caryophyllene	1451	1419	2.07±0.29^{b}	2.60±0.14^{b}	1.49±0.25^{a}	1.09±0.09^{a}
Germacrene D	1513	1481	2.71±0.05^{c}	3.67±0.14^{d}	1.91±0.02^{b}	0.69±0.04^{a}
Spathulenol	1596	1578	1.71±0.0^{c}	2.15±0.09^{d}	1.00±0.06^{b}	0.58±0.06^{a}
Caryophyllene oxide	1605	1583	0.68±0.06^{a}	1.12±0.04^{b}	1.01±0.03^{b}	2.21±0.37^{c}
Oplopanone	1752	1734	2.54±0.05^{a}	3.48±0.61^{b}	3.19±0.21^{b}	5.86±0.77^{c}
Heptacosane	2711	2700	3.11±0.10^{b}	3.35±0.17^{b}	2.47±0.32^{a}	3.71±0.18^{c}
Squalene	2823	2790	7.06±0.12^{c}	2.16±0.15^{a}	4.70±0.03^{b}	-
Farnesol isomer a	2832	n.a	2.08±0.25^{a}	2.97±0.17^{c}	2.42±0.05^{b}	2.81±0.23^{c}
6-methyloctacosane	2854	2846	3.22±1.49^{b}	2.83±0.61^{b}	2.01±0.26^{a}	2.25±0.23^{a}
Hexacosane	2911	n.a	6.13±1.13^{a}	7.52±0.36^{b}	5.58±0.74^{a}	9.42±0.86^{c}
n-Tetracosanol-1	2926	n.a	18.30±0.71^{c}	12.45±0.54^{a}	14.33±1.09^{b}	18.05±1.28^{c}

Referred average values of six determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$) KI* - Kováts retention indices calculated against C8–C32n-alkanes, KI** - Kovats indices reported in literature (Adams, 2009)

α-Terpineol amount in extracts obtained after Soxhlet was also higher than after SFE-CO_2 (1.2-1.9% w/w, 0.5% w/w, respectively). Geraniol amount in Soxhlet extracts was 4.3-7.3% w/w, in SFE-CO_2 only 1.7% w/w [70]. Trans-β-ocymene amounts in hexane-soluble Soxhlet extracts was 2.3-2.9% w/w, in SFE-CO_2 – 7.3% w/w and in ethanol soluble extracts only 0.5-0.7 % w/w. Comparing these authors work and results obtained in this study 6 of same compounds were identified: α-thujene, sabinene, β-myrcene, trans-β-ocymene, α-terpineol, spathulenol. Stamenic et al (2014) identified the following compounds in extracts obtained after SFE-CO_2 (10MPa, 40°C, 30MPa, 40°C, 30MPa, 40°C): carvacrol (58.3-79.9%), thymol (2.6-5.7%), citronellyl formate (4.0-12.9%), p-cymene (0.7-2.8%), trans-β-caryophyllene (1.1-1.8%), β-bisabolene (0.8-1.4%) [74]. Comparing constituents identified after conventional Soxhlet extraction with those identified after high pressure SFE-CO_2 extraction were reported the same compounds such as bourbonene, caryophyllene, caryophyllene oxide, limonene, squalene, oplopanone. Due to presence of well-known antioxidant squalene in all isolated extracts, there was decided to investigate squalene presence and amounts in most of obtained extracts after various extractions.

Table 30. Compounds identified by GC-MS in SFE-CO_2 extracts

Compound			SFE-CO_2							
	KI*	KI**	25°C	40°C	50°C	70°C	40°C+EtOH	50°C+EtOH	70°C +EtOH	HD
Limonene	1048	1045	2.51±0.31c	0.22±0.03a	-	-	3.11±0.06d	-	1.05±0.12b	3.47±0.64d
α-Bourbonene	1405	1388	0.48±0.01a	0.72±0.08b	0.71±0.21b	1.33±0.05c	-	0.70±0.08b	-	-
E-Caryophyllene	1446	1419	0.55±0.04a	1.38±0.12b	1.16±0.22b	1.88±0.22c	-	1.05±0.16b	-	-
Spathulenol	1599	1578	0.79±0.03a	1.42±0.17b	1.80±0.32c	1.86±0.06c	0.66±0.02a	1.70±0.22bc	1.55±0.13b	-
Caryophyllene oxide	1608	1583	1.55±0.04a	1.28±0.15a	2.14±0.52b	2.52±0.12c	1.35±0.05a	2.03±0.15b	1.63±0.07a	-
Oplopanone	1754	1734	5.82±0.27c	5.88±0.61c	7.29±1.17e	7.11±0.19e	4.98±0.2b	6.85±1.35	6.55±0.33d	3.39±0.42a
Phytol acetate	1838		0.88±0.05a	0.97±0.07a	1.9±0.14c	1.29±0.15b	5.80±0.97e	0.97±0.07a	2.87±0.32d	1.25±0.26b
Phytol	2129	2111	0.74±0.13a	0.56±0.08a	-	-	1.01±0.09b	-	1.16±0.94b	3.22±0.34c
Oleamide	2389	2375	0.62±0.01b	1.69±0.18d	1.07±0.08c	1.19±0.28c	1.86±0.07d	0.73±0.06b	0.24±0.00a	1.74±0.22d
Hexacosane	2498	n.a	1.10±0.07a	1.19±0.16ab	1.29±0.17ab	1.13±0.18a	0.99±0.08a	1.62±0.06c	1.52±0.30c	1.11±0.11a
Octacosane	2597	n.a	1.86±0.10c	0.29±0.02a	-	-	1.47±0.09b	-	3.42±0.41d	2.03±0.20c
Heptacosane	2697	2700	4.69±0.49b	3.77±0.23a	4.20±0.52b	3.53±0.11a	4.38±0.27b	4.46±0.38b	5.09±0.86c	4.58±0.49b
2-methyloctacosane	2769	n.a	4.16±0.38c	2.53±0.19a	3.29±0.38b	2.78±0.15a	4.29±0.49c	3.62±0.36b	4.95±0.71d	4.72±0.60d
Squalene	2817	2790	3.05±0.17b	3.46±0.08c	3.71±0.56d	3.44±0.44c	2.34±0.15a	4.18±0.65	2.87±0.09b	3.34±0.12bc
6-methyloctacosane	2848	2846	-	1.21±0.34a	1.19±0.14a	1.12±0.16a	-	1.22±0.11a	-	-
Heptacosane	2857	n.a	-	1.00±0.08a	1.28±0.24b	1.01±0.13a	-	1.25±0.11b	-	-
Docosane 11-butyl-	2897	n.a	-	8.86±1.27a	10.71±1.08b	8.74±0.31a	-	10.30±0.72b	-	-
1-Hexacosene	2912	n.a	13.95±1.71b	42.14±3.54f	37.59±6.56e	33.44±0.98d	11.90±0.56a	29.50±4.72c	10.77±0.30a	12.37±1.41ab
1-Docosane	2926	n.a	33.18±2.02	-	-	-	33.48±6.01	-	18.81±2.56	38.41±2.79
2-methyloctacosane	2968	n.a	-	3.64±0.36a	4.02±0.69b	3.36±0.18a	-	4.09±0.48b	-	-
Sulfurous acid. butyl dodecyl ester	2984	n.a	5.28±0.55a	7.00±0.18c	7.64±0.43d	6.14±0.66b	4.58±0.68a	7.06±0.58c	4.93±0.64a	4.46±0.76a

Referred average values of six determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$) KI* - Kováts retention indices calculated against C8–C32n-alkanes, KI** - Kovats indices reported in literature (Adams, 2009)

3.5.4. Squalene determination by HPLC

Squalene is a polyunsaturated triterpene, common in nature could be animal-derived (shark liver), plant-derived (olive oil, wheat germ, rice bran, amaranth seeds) [13,180] and synthetic [181]. Shark liver is considered as a major source for squalene, however sharks population decreased and have attracted attention as an endangered species [182]. Squalene production from plants, microorganisms such as yeasts did not give expected results, because these squalene contents have been very low for commercial use [183]. In humans, squalene is synthesized in the liver and the skin, transported in the blood by the small and very small density lipoproteins [180]. Squalene has well-known antioxidant activity which could be explained by its structure: squalene is an isoprenoid, containing 6 double bonds (**Fig. 24**) [184]. Squalene amount in different samples was obtained using HPLC analysis and selected chromatograms are presented below (**Fig. 24**).

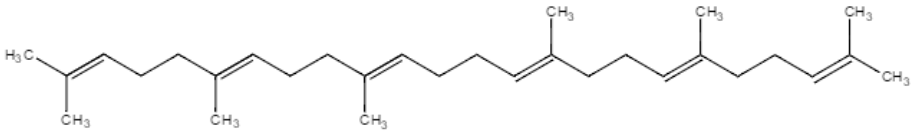

Fig. 24 Squalene

The content of squalene in the isolated constituents and measured by HPLC varied from 1.3 to 218.8 μg/g DW. The lowest squalene amount observed in SLE-He, PLE-He samples (1.3, 1.9 μg/g DW, respectively). 1.4-3.8-fold higher squalene content were found in samples after SFE-CO_2 at 25°C, 40°C and 50°C and 70°C HD. Ethanol modifier increased squalene amount in isolated constituents up to 4-fold. Using acetone as solvent in SLE even at lower temperature (40°) resulted highly increased squalene amount, up to 2.9-5.6-fold. In polar extracts after PLE with ethanol and Soxhlet with acetone squalene amount increased 2.8-4.2-fold. The highest squalene amount was obtained in extracts isolated after PLE with acetone, and resulted 218.9 μg/g DW. From obtained results could be seen that higher squalene amounts were isolated using polar solvents (acetone, ethanol, also in SFE-CO_2 extraction ethanol increased polarity and obtained squalene amount was higher), temperature did not have high impact on obtained squalene amount. As in previously discussed oxipres results, it was observed that acetone-soluble PLE extract increased rapeseed oil induction period the most, now it could be concluded, that these results have impacted high squalene amount in this extract.

Table 31. Squalene amount in different extracts

Sample	Extraction conditions	Squalene amount	
		µg/g extract	m*g/kg DW*
Non-polar extracts			
Conventional extractions:			
SLE-He	T-50°C, τ-360 min	75.38±1.22[a]	1.34±0.02[a]
Soxhlet-He	T-69°C, τ-360 min	108.38±4.05[b]	3.48±0.13[b]
High pressure extractions:			
SFE-CO_2	T-25°C, τ-210 min, P-45 MPa	205.99±13.47[c]	2.70±0.18[b]
SFE-CO_2	T-40°C, τ-210 min, P-45 MPa	234.81±9.64[d]	3.55±0.15b[c]
SFE-CO_2	T-50°C, τ-210 min, P-45 MPa	201.75±9.36[c]	4.06±0.19[c]
SFE-CO_2	T-70°C, τ-210 min, P-45 MPa	218.99±8.38c[d]	5.04±0.19[d]
SFE-CO_2 (+5% EtOH)	T-40°C, τ-210 min, P-45 MPa	362.66±30.86[e]	10.59±0.90[f]
SFE-CO_2 (+5% EtOH)	T-50°C, τ-210 min, P-45 MPa	458.30±42.07[f]	14.34±1.32[g]
SFE-CO_2 (+5% EtOH)	T-70°C, τ-210 min, P-45 MPa	568.96±29.70[g]	15.70±0.82[g]
HD SFE-CO_2	T-70°C, τ-210 min, P-45 MPa	367.13±29.48[e]	7.34±0.69[e]
PLE-He	T-50°C, τ-15 min	63.04±4.13[a]	1.94±0.13[ab]
Polar extracts			
Conventional extractions:			
SLE-Ac	T-40°C, τ-360 min	1996.72±33.70[i]	40.25±0.0,69[h]
Soxhlet-Ac	T-56°C, τ-360 min	2764.79±55.66[k]	173.63±3.50[j]
High pressure extractions:			
PLE-EtOH	T-120°C, τ-30 min, P-10 MPa	760.12±52.73[h]	115.01±7.98[i]
PLE-Ac	T-120°C, τ-30 min, P-10 MPa	2515.68±153.45[j]	218.86±13.35[k]

Referred average values of three determinations ± SD. Different superscript letters within the columns indicate significant differences (one way ANOVA, Tukey's test $p<0.05$)

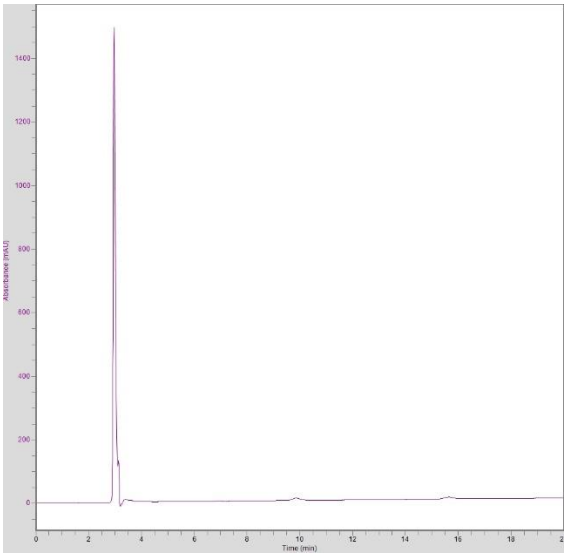

Fig. 25 a) Squalene standard (3µg/ml)

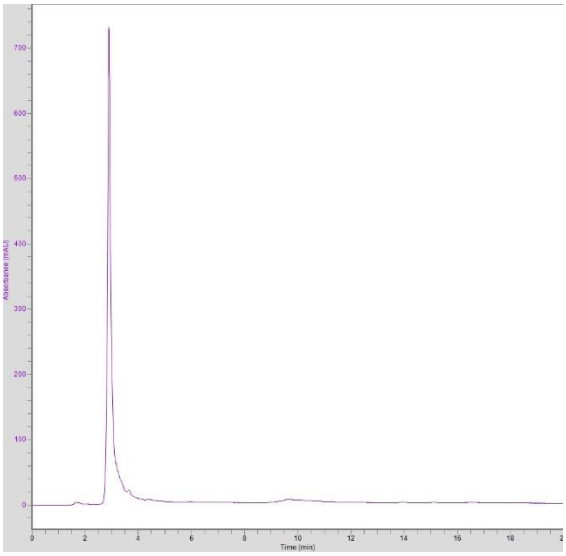

Fig. 25 b) Squalene in PLE-Ac extract

. Squalene is a component of adjuvants, which enhances the body's immune response to antigen that is added to vaccines. The vaccine contains of 10 mg squalene per dose [172]. Squalene inhibits developments of various tumors. It is well tolerated whether injected intravenously or consumed orally. Orally administrated squalene is absorbed 60-85% [173]. Squalene is believed to be partially responsible for the low number of human cancer in Mediterranean region because peoples diet has high amount of olives [174]. This compound is also believed to protect the skin from ultraviolet radiation-induced damage, because a high percentage of squalene is secreted in sebum.

In animals models, also been shown exhibit radioprotective properties. This natural compound accumulates in the skin, where it is responsible of quenching oxygen singlet, thus preventing harmful effects of lipid peroxidation [173,175]. Although some controversy still exists following vaccination using squalene-based formulations, recent publications describing the use of validated assays have clarified, to some extent, the safety of squalene as an adjuvant [173]. Nevertheless, it is important to exercise caution when developing squalene-based formulations for human use.

4. CONCLUSIONS

1. The following chemical composition and *in vitro* antioxidant activity indices were determined for oregano, used for the purposes of this study: lipid content 7.6 g/100g, protein content 10.1 g/100g, mineral content 7.1 g/100g, moisture content 5.5 g/100g, total phenolic content 116.6 mg GAE/g DW, $ABTS^{\bullet+}$ scavenging capacity 403.7 mg TE/g DW, $DPPH^{\bullet}$ scavenging capacity 162.4 mg TE/g DW and oxygen radical absorbance capacity 529.2 mg TE/g DW. The obtained results indicate that plant material can be considered as a promising source of natural antioxidants with particular reducing properties and antiradical capacity *in vitro*.
2. According to the volatile compound composition in essential oil, oregano, used for the purposes of this study, was assigned to sabinene/germacrene D chemotype: sabinene (8.1-33.2%), germacrene D (6.8-12.3%), α-caryophyllene (5.9-11.6%), *E*-α-ocimene (3.2-9.6%), α-phellandrene (2.5-4.7%). By means of hydrodistillation (100°C, 240 min), the highest yield of essential oil was obtained from unground fraction (0.21 ml/100 g DW), while 14-38% reduction was obtained reducing particle size of plant material: 1 mm (0.18 ml/100g DW) > 0.5 mm (0.15 ml/100g DW) > 0.2 mm (0.13 ml/100g DW). Particular differences in volatile compound profile were observed too. Sabinene was found at the highest percentage in ungroud fraction, *E*-α-ocimene and α-phellandrene – in 1 mm fraction, while α-caryophyllene and germacrene D – in 0.2 mm fraction.
3. At the 1st step, optimal SFE-CO_2 conditions for oregano plant material were determined (45 MPa, 50°C and 210 min), yielding 2.0 g/100 g DW of SFE-CO_2 extract. The addition of 5% EtOH modifier further improved extraction yield by ~56% (3.1 g/100g DW). In continuation, PLE protocol with different polarity food-grade solvent (acetone, EtOH, EtOH/H_2O and H_2O) was developed to extract semi-polar and polar fractions from oregano residue after SFE-CO_2. At the 2nd step, optimized PLE-Acetone conditions (120°C, 30 min) yielded 8.7 g/100g of acetone-soluble extract with TPC value of 56.0 mg GAE/g DW. Further sequential solid residue extraction with EtOH and H_2O (3rd and 4th) steps recovered 15.5g/100 of ethanol-soluble (TPC: 70.6 mg GAE/g DW) and 22.7 g/100g DW of water-soluble constituents (TPC: 51.6 mg GAE/g DW), respectively. The usage of EtOH/H_2O mixure at the 3rd step resulted in 9% and 21 % lower global PLE yield and total phenolic content (after all steps of extraction), respectively.
4. Using conventional extraction techniques (Soxhlet, SLE), yield of non-polar constituents varied in range from 1.8 to 3.3 g/100g DW with TPC values from 0.6 to 0.9 mg GAE/g DW and was 1.1-2.5-fold higher than non-polar extracts isolated by high-pressure extraction techniques (SFE-CO_2 and PLE). Polar extract yields after conventional extractions varied from 1.6 to 19.1 g/100g DW with TPC values from 2.3 to 72.0 mg GAE/g DW, while application

of PLE for polar oregano constituent removal resulted in ~52% higher yields with up to 3-fold higher TPC content at 3-8-fold shorter extraction times.

5. Antioxidant capacity of non-polar and polar constituents of oregano was in the range of: TPC=0.5-88.0 mg GAE/g DW, $TEAC_{DPPH}$=0.12-127.7 mg TE/g DW, $TEAC_{ABTS}$=0.25-295.8 mg TE/g DW, $TEAC_{ORAC}$=1.5-456.6mg TE/g DW, with the highest activities noted for PLE extracts (up to 90-fold, as compared to conventional extraction techniques). The total activity of oregano extracts by sequential SFE-CO_2 and PLE (Acetone → EtOH → H_2O) under the optimized conditions was 178.7 mg GAE/g DW, 424.3 mg TE/g DW (ABTS), 164.1 mg TE/g DW (DPPH) and 1154.5 mg TE/g DW (ORAC). This high-pressure fractionation scheme reduced the initial total phenolic content and antiradical capacity of starting plant material before SFE-CO_2 by 94-98%, showing its efficiency to remove the major portion of antioxidatively active constituents from oregano.
6. The addition of 1% (w/w) PLE-Acetone (120°C, 30 min) extract to rapeseed oil increased its oxidative stability by 66% (induction period of 6.46 h versus 3.88 hours of control sample). Other tested extracts (SFE-CO_2 and PLE-EtOH) at this concentration range had less pronounced effect, showing only ~10% increase in oxidative stability of oil.
7. In non-polar oregano extracts (Soxhle-He, SLE-He, SFE-CO_2, PLE-He), 16 compounds (mainly, fatty acids and phenolic compounds) were tentatively identified, mainly originating from SFE-CO_2 (50°C, 210 min, 45 MPa) and SFE-CO_2 (50°C, 210 min, 45 MPa, +5% EtOH) extracts. Main identified fatty acids were palmitic (11.0-21.4%), linoleic (9.7-11.5%) and heneicosanoic (20.0-38.8%), with no variations due to the conventional or high pressure extraction techniques applied. Other identified compounds in non-polar extracts were n-tetracosanol-1 (12.45-18.30%), hexacosane (5.58-9.42%), 1-hexacosene (10.77-42.14%), 1-docosane (18.81-33.48%), α-thujene (1.09-9.19%), α-pinene (1.03-8.01%) and squalene (4.70-7.06%). In semi-polar and polar oregano extracts (Soxhlet-Ac, SLE-Ac, aqueous extracts after hydrodistillation, PLE-Ac/EtOH/H_2O), 28 compounds were tentatively identified, mainly belonging to phenolic acids and other phenolic compounds. Squalene content in hexane, SFE-CO_2 and acetone extracts varied from 1.3 to 3.5 mg/kg DW, from 1.9 to 15.7 mg/kg DW, and from 40.3 to 218.9 mg/kg DW, respectively with PLE-Ac being the most efficient extraction technique for squalene isolation from oregano.

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