„DUNĂREA DE JOS" UNIVERSITY OF GALAŢI

FACULTY OF FOOD SCIENCE AND ENGINEERING

PhD thesis

OBTAINING AND CHARACTERIZATION OF SOME VEGETAL EXTRACTS WITH BIOLOGICALLY ACTIVE POTENTIAL FROM AROMATIC PLANTS (Abstract)

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Către

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CONTENT

II. ORIGINAL CONTRIBUTIONS AND PERPECTIVES

Scientific objectives

The need for clean processes and safe products is the essence of green chemistry which is linked to environmental protection and the demand for quality and value added products.

These processes apply to all sectors of economic activity and are one of the main European Union strategies to protect the environment. The concern in the European Union for sustainable development issues led to the adoption of processes which are environmentally friendly, in order to help to reduce waste and environmental pollution through the exploitation of natural resources and energy.

Traditional extraction methods used to obtain these types of products have several drawbacks; they are time consuming, laborious, low selectivity and/or low extraction yields. Moreover, these traditional techniques employ large amounts of toxic solvents.Nowadays, extraction methods which are able to overcome the above mentioned drawbacks are being studied (*e.g.Supercritical fluid extraction*).

Supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) are some of the more promising processes. These extraction techniques provide higher selectivity, shorter extraction time and do not use toxic organic solvents.

Use of supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) has opened a modern and extensive research field with practical application in many fields such as food industry, agricultural products processing, pharmaceutical and nutraceutical industry, cosmetics etc.

In the last years, many research publications were dedicated to so-called "functional foods" and "nutraceuticals". Research on functional ingredients was showing promising prospects regarding the utility of such ingredients in food products, thereby improving the for benefits for consumer health and for the manufacturers.

The term "functional food" was coined in Japan in the early 1980s describing the products that were meant to reduce the high costs in the provision of health and life quality. A functional food is one that can provide additional physiological benefit, other than energy and nutrition (Goldberg, 1996). Based on this definition, currently

the attribute "functional" means food that induce beneficial effects for one or more physiological functions, increase wellness and/or reduce the risk of certain diseases (Guarneri and Azpiroz, 2005).

This definition includes three important aspects:

1) the functional effect is different than the nutritious
2) the functional effect must be demonstrated satisfactorily
3) the benefit can consist in an improvement of a physiological function or in a reduction of risk of developing a pathological process.

Frequently, functional foods are obtained from traditional foods enriched with an ingredient able to provide or promote a beneficial action for human health. These are the so-called functional ingredients.

These ingredients are preferred by consumers to have a natural origin (*i.e.* non synthetic origin) being commonly extracted from natural sources such as plants, food by products or even algae and microalgae.a

Bioactive compounds responsible for the beneficial effect may be present naturally in functional foods, or can be formed or added in the product during processing. Therefore, there were developed two terms, depending of the incorporation conditions: *product enriched* when bioactive compounds increases by increasing the original that are found in small quantities and *fortified product* when supplemented compounds were not present in the original food.

PhD thesis entitled: **Obtaining and characterization of some vegetal extracts with biologically active potential from aromatic plants** has the main objective of the study bioactive potential of aromatic plants from spontaneous flora of Romania, oregano, wild thyme and tarragon by applying modern and efficient methods of extraction, chemical characterization of extracts and evaluation of physiological properties (antioxidant capacity, antimicrobial activity and prebiotic effect).

In this context, the doctoral program studies focused on the following objectives:

- Study of bioactive potential of aromatic plants from spontaneous flora of Romania (oregano, wild thyme and tarragon) by applying modern extraction techniques, pressurized liquid extraction (PLE) (with different solvents and

different temperatures) and supercritical fluid extraction (SFE) as sample preparation technique for the analysis of target compounds from natural products and foods.

- *In vitro* characterization of bioactive properties of extracts by evaluating total polyphenol content, antioxidant capacity, antimicrobial activity and prebiotic effect.
- Chemical characterization of the obtained extracts by chromatography (HPLC and GC) coupled with mass spectrometry (MS) to identify bioactive compounds.
- Stability and solubility evaluation of bioactive compounds from the obtained extracts and delivery for use in food systems by microemulsions.

For carrying out the research, according to the scientific objectives of the PhD thesis, a modern research infrastructure was used:

- Integrated Research and Formation Center for Apllied Biotechnology in Food Industry - Bioaliment Platform (www.bioaliment.ugal.ro), Faculty of Food Science and Engineering, "Dunarea de Jos" University of Galati
- FOODOMICS Laboratories, Institute of Food Science Research, Madrid, Spain

PhD THESIS STRUCTURE

The PhD manuscript is presented on 164 pages and it is divided into two parts as follows: **I. Literature review**, which contains 4 chapters and **II. Original contributions and perspectives** structured on 7 chapters. The manuscript contains 62 figures and graphs and 7 Tables.

I) **LITERATURE REVIEW**, is divided in four chapters with the following topics:

- **Chapter 1**, entitled **Bioactive compounds from plants and the impact on life quality** is divided in two subchapters that describe the main categories of bioactive compounds, their classification and how they act *in vivo* to improve the consumers' health.
- **Chapter 2**, entitled **Aromatic plants of autochthonous flora: taxonomy and phytochemical composition** is divided into three subchapters, describing the plants used to obtain bioactive compounds. It present a description of the characteristics of these plants in order to identify them as well as the geographical area where they naturally grow (spontaneous flora). Furthermore, are given references to relevant literature data for the phytochemical composition of plants used for extraction of bioactive compounds.
- **Chapter 3**, entitled **Modern extraction techniques and characterization of bioactive compounds from plants** is divided into four subchapters. The first two subchapters describe the main modern extraction techniques of bioactive compounds such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). The next two subchapters contain a description of separation and identification of bioactive compounds.
- **Chapter 4**, entitled **Solubility, stability and delivery of bioactive compounds in food systems by microemulsions** is refering to the importance of microemulsions on the solubility and stability of bioactive compounds and also the contribution of microemulsions to deliver

bioactive compounds in food. It also described the main components of microemulsions, the importance and role of each component for obtaining and stabilizing of microemulsions.

II) **ORIGINAL CONTRIBUTIONS AND PERSPECTIVES** cover the original results obtained in this study and it is divided in seven chapters, as it follows:

- **Chapter 5**, entitled **Preliminary study of obtaining and functional characterization of some vegetal extracts from aromatic plants** presents the results obtained by a classic method of bioactive compounds extraction from oregano (*Origanum vulgare* L.), tarragon (*Artemisia dracunculus*) and wild thyme. Characterization was performed to determine the total phenolic content by Folin-Ciocâlteu method and the antioxidant capacity, using photochemiluminiscence method (PCL).
- **Chapter 6**, entitled **Chemical and functional characterization of essential oils from aromatic plants obtained by supercritical fluid extraction (SFE)** presents the results of the study concerning the obtaining and characterization of essential oils from oregano and wild thyme. The optimization of extraction conditions was carried out using the Taguchi experimental design. For evaluation of the functional properties of essential oils, total phenolic content, antioxidant capacity (DPPH method) and antimicrobial activity were analyzed. Separation and identification of bioactive compounds were performed by gas chromatography coupled with mass spectrometry (GC-MS).
- **Chapter 7**, entitled **Chemical and functional characterization of bioactive pressurized extracts of Romanian aromatic plants** presents the results of the study for obtaining and characterization of bioactive pressurized extracts from oregano, tarragon and wild thyme. Different PLE conditions were tested including extraction with water, ethanol and their mixtures in a wide range of extraction temperatures (50-200 °C). PLE was used as green and sustainable extraction technique while functional characterization was carried out by using different *in-vitro* assays, including total phenols determination as well as two different antioxidant

capacity assays (DPPH and TEAC), antimicrobial activity and prebiotic effect. Moreover, extracts were chemically characterized by using a LC-MS/MS method to correlate the antioxidant activities with the particular chemical composition.

- **Chapter 8**, entitled **Studies of solubility, stability and delivery of bioactive compounds from aromatic plants** presents the results obtained using microemulsions as solubilization and stabilization systems of oregano and wild thyme extracts obtained by PLE. The solubility of the extracts was tested in different vegetable oils. In order to obtain and stabilize the microemulsions, different ratios of vegetable oil were used: surfactant: cosolvent. PLE extracts were solubilised in microemulsions and were delivered in fresh pear juice, with the aim to improve the antioxidant capacity. The results were compared with those obtained by adding two synthetic antioxidants: butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).
- **Chapter 9** covers the **General conclusions** which point out the scientifical characteristics and the application of the PhD thesis focused on obtaining, characterization and evaluation of biologically active extracts of aromatic plants of spontaneous flora from Romania.
- **Chapters 10** and **11** summarize the original contributions of the PhD research stage, the impact on the knowledge development, perspectives for future research, as well the the results dissemination.

II. ORIGINAL CONTRIBUTIONS AND PERSPECTIVES

5. Preliminary study of obtaining and functional characterization of some vegetal extracts from aromatic plants

5.2. Materials and methods

Plant samples

Three plant materials dried, belonging to three botanical families, which are commonly consumed in Romania, were chosen for this study: oregano (*Origanum vulgare*), tarragon (*Artemisia dracunculus*) and wild thyme (*Thymus serpyllum*). The plant samples were obtained from a herbalist's shop in Galati, Romania.

Solvents and chemicals

Gallic acid and ethanol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Standard Folin-Ciocalteu's phenol reagent and anhydrous sodium carbonate were obtained from Merck (Darmstadt, Germany). ACL (Antioxidant Capacity of Liposoluble substance) kits, Trolox ((S)-(2)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were purchased from Analytik Jena AG, Jena, Germany. Ultra pure water was used for the experiments.

5.2.1. Phenolic compounds extraction

Dry plants were extracted by varying concentrations (0-95%) of ethanol. Among various extracts the highest phenolic containing extracts (70% ethanol) were chosen for further analysis. 10 g of plant were brought to 100 mL 70% ethanol. The mixture was left 24 hours for maceration at room temperature (20 °C) and then put in an ultrasonic bath at 60 °C, 2 h. The suspension was then vacuum filtered through a ceramic filter with the porosity of 40 μm and centrifuged at 10,000 rpm, 15 min. The ethanol was completely removed by vacuum concentrator (Martin Christ), at 50 °C to give a viscous mass. The crude extracts were stored at 0-4 °C before analysis.

5.2.2. Determination of the Antioxidant Capacity of Lipid-Soluble (ACL) Compounds by the Photochemiluminescence (PCL) Method

PCL analysis for determining the antioxidant capacity of the plant extracts was carried out with a Photochem apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photosensitizer, when exposed to UV light. The antioxidant capacity of 70% ethanol extracts of plants was measured using ACL kits provided by the manufacturer designed to measure the antioxidant activity of lipophilic compounds. In ACL studies, the kinetic light emission curve, which exhibits no lag phase, was monitored for 180 s and expressed as micromoles of Trolox per gram of dry matter. The areas under the curves were calculated using PCLsoft control and analysis software. As greater concentrations of Trolox working solutions were added to the assay medium, a marked reduction in the magnitude of the PCL signal and hence the area calculated from the integral was observed. This inhibition was used as a parameter for quantification and related to the decrease in the integral of PCL intensities caused by varying concentrations of Trolox. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added extract solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve.

5.2.3. Determination of total phenolic content by Folin-Ciocalteu method

The total phenolic content (TPC) was determined by spectrophotometry, using gallic acid as standard, according to the method described by the International Organization for Standardization (ISO) 14502-1. Briefly, 1.0 mL of diluted sample extract was transferred in duplicate to separate tubes containing 5.0 mL of 1/10 dilution of Folin- Ciocalteu's reagent in water. Then, 4.0 mL of sodium carbonate solution (7.5% w/v) was added. The tubes were allowed to stand at room temperature for 60 min before absorbance was measured against water at 765 nm. The TPC was expressed as gallic acid equivalents (GAE) in g/100 g material. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 10 to 50 μg/mL.

5.3. Results and discussion

The purpose of this study was to quantify and compare the antioxidant capacities of three plants from Romania. Indeed, an increasing interest in the health benefits of plants has led to the inclusion of plant extracts in dietary supplements and functional food.

Oregano (*Origanum vulgare*), tarragon (*Artemisia dracunculus*) and wild thyme (*Thymus serpyllum*) accumulate large amounts of phenolic compounds. In accordance with the dry weight of the extract, the total phenolic content of the studied plant extracts decreased as follows: oregano (*Origanum vulgare*) > tarragon *(Artemisia dranunculus)*> wild thyme *(Thymus serpyllum)* (fig. 5.3)

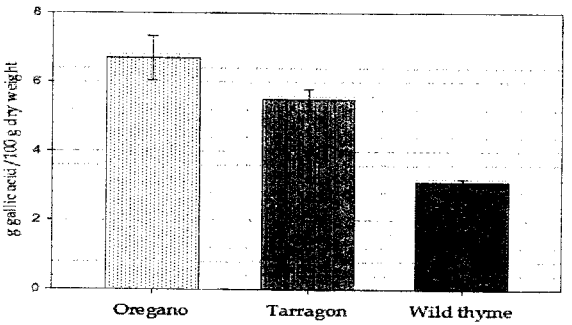


Figure 5.3. Total phenolic content of ethanolic extracts

Photochem® apparatus and method allow a precise determination of the antioxidant capacity of lipid-soluble (ACL) compounds and are efficient in time and cost (Apati et al., 2003). Free radicals are generated in the measuring system itself by means of photosensitizer. The free radicals were detected by their reaction with the chemiluminogenic substance. Luminol played double role as photosensitizer and also as oxygen radical detection reagent. In the presence of substances in plant extracts which act as radical traps, the intensity of the PCL was attenuated according to the concentration. In this way, the antiradical properties of the analyzed substances could reliably be quantified.

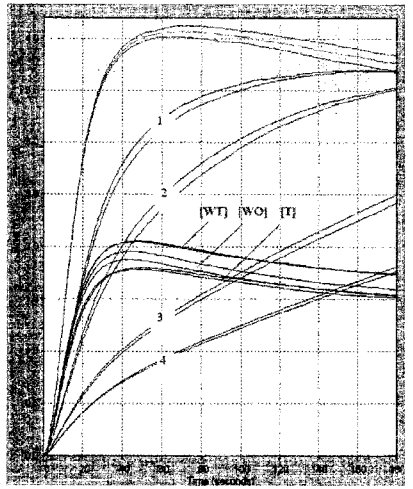


Figure 5.4. Antioxidant activity of lipid-soluble components of three plant extracts as measured by photochemiluminescence.
[WT] -wild thyme, [WO] - oregano and [T]-tarragon. 1-4 are trolox standards: 5, 10, 20, 30 µl.

Antioxidant activities measured using PCL methods are shown in figure 5.4 and figure 5.5. The results for aromatic plants are presented in equivalent concentration units of trolox (figure 5.5).

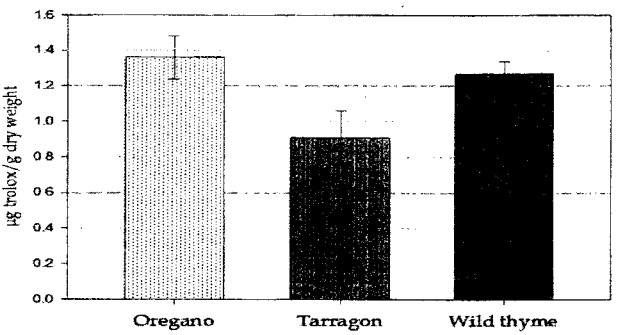


Figure 5.5. Lipid soluble antioxidant capacity determined by photochemiluminiscence of oregano, tarragon and wild thyme ethanolic extracts

Differences between the results were likely due to genotypic and environmental differences (climate, location, temperature, fertility, diseases and pest exposure) within species, choice of parts tested, time of taking samples.

The strong antioxidant activity of oregano (*Origanum vulgare*) provided from Romania was expected on the basis of the literature (Exarchou, Nenadis, Tsimidou, Gerothanassis, Troganis & Boskou, 2002), (Wojdylo et al., 2007), (Shan, Cai, Sun & Corke, 2005), (Ivanova, Gerova, Chervenkov & Yankova, 2005).

Our results showed that oregano (*Origanum vulgare*), tarragon (*Artemisia dracunculus*) and wild thyme (*Thymus serpyllum*) were rich in phenolic constituents and had good antioxidant capacity. The results prove the importance of phenolic compounds in the antioxidant behavior of spice extracts and also that they contribute significantly to the antioxidant activity of lipid-soluble components. Therefore, qualitative and quantitative analysis of major individual phenols in the spices could be helpful in explaining the relationships between total antioxidant capacity and total phenolic contents in the species.

5.4. Conclusions

The total phenolic content and antioxidant capacity of three different plants from Romania were measured. The results showed the high phenolic content and antioxidant capacity and provide useful information like the potential use of plants as a natural source of antioxidants and as a value-added product in the preparation of functional food ingredients and/or for enrichment of certain products.

Providing a reliable analytical method to detect the antioxidant capacities at very low concentrations of antioxidant in the sample is of major interest. Photochemiluminescence analysis provides many advantages over the other methodologies. It is simple, quick, sensitive, economical, convenient, and reliable.

The results support the possibility that these plants, which are commonly used in the Romanian diet as condiments or decoctions, can show protective effects on human health. However, further *in vivo* investigations on the absorption and metabolism of plants' bioactives are still necessary to further shed light on their efficacy for disease risk reduction.

6. Chemical and functional characterization of essential oils from aromatic plants obtained by supercritical fluid extraction (SFE)

6.2. Materials and methods

The plant samples were obtained from a herbalist's shop in Galati, Romania. Samples were stored at 4°C until use for extraction.

Ethanol absolute was from Prolabo (Madrid, Spain). Ultrapure water quality (18.2 MΩ cm) with 1-5 ppb total organic carbon (TOC) and <0.001 EU/mL pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica,USA).

1,1-Diphenyl-2-picrylhydrazyl hydrate (DPPH, 95% purity) was obtained from Sigma-Aldrich (Madrid, Spain), ethanol from VWR BDH Prolabo (Madrid, Spain) and methanol from Panreac Quimica (Barcelona, Spain). Folin-Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) whereas antioxidant standards, i.e. gallic acid was supplied by Sigma-Aldrich (Steinheim, Germany). Carbon dioxide (CO2) liquefied at high pressure used in supercritical fluid extraction was supplied by Praxair (Madrid, Spain). Washed glass wool chemically pure was acquired from Panreac.

Dimethyl sulfoxide (DMSO), purity 99.9% was purchased from Fluke (Switzeland).

Escherichia coli ATCC 25922 was used for evaluation of the antimicrobial activity of essential oils obtained by supercritical fluid extraction (SFE).

6.2.1. Supercritical fluid extraction (SFE)

The supercritical extraction apparatus used to perform the experiments is schematically presented in Fig. 6.2. The equipment is based on a Suprex Prep Master (SuprexCorporation,Pittsburg, PA,USA) with several modifications. It has a thermostatic oven heated by air convection where the extraction cell (with approximately 10 cm3 of internal volume) containing the sample is placed. A pre-heater system was employed by placing a heating coil inside a glycerin bath (JP Selecta Agimatic N ,JP Selecta S.A., Abrera, Spain) to guarantee that the fluid employed in all the experiments reaches the high pressure vessel at the target temperature. The system is also equipped with a Suprex solvent modifier pump.

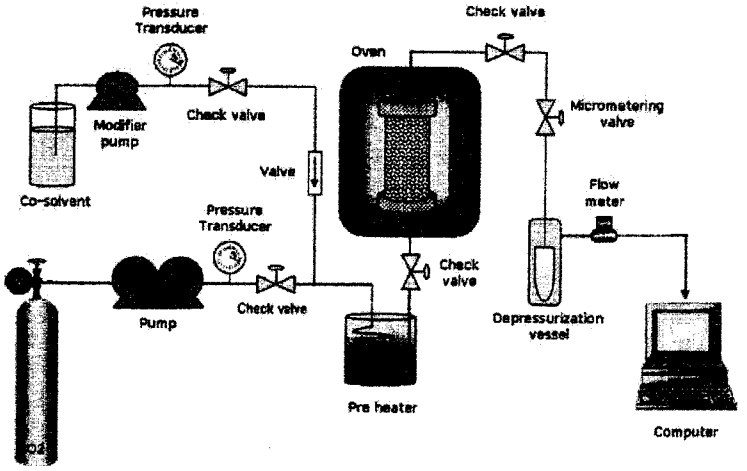


Figure 6.2 Schematic diagram of the supercritical fluid extraction apparatus used in this work. The equipment consists in a heated high pressure cell in which scCO2 liquefied +co-solvent is introduced, followed by a depressurization system.

After the modifier pump, a check valve (Swagelok SS-CHS2-BU-10, Swagelok Corporation, Solon, OH, USA) was used. Another Swagelok check valve and a micrometering valve (Hoke SS-SS4-BU-VH, Hoke Incorporated, Spartanburg, SC, USA) were placed after the extraction cell to manually control the flow. A linear restrictor consisting on a silica capillary (50cm×75 μm i.d.) was used to control slow decompression of the system. Carbon dioxide flow rate was measured by a computer-controlled mass flow meter (EL-FLOW MassFlowMeter/Controller F-111C,Bronkhorst High-Tech BV, AKRuurlo, TheNetherlands).

6.2.5 Gas chromatography –mass spectrometry (GC-MS) analysis of essential oils

The bioactive compounds from essential oils were analyzed with a Shimadzu QP2010 GC system coupled to a quadrupole mass spectrometry. The column used in the GC was a fused silica capillary column (inside diameter, 30 m by 0.25 mm) coated with a 0.25 μm layer of SE-54 (Quadrex Corp. Woodbridge, USA). The method used was formerly described by Herrero *et al.*, 2006. The injection was carried out at 250 °C in split mode (ratio 1:20). The volume of injection was 10 μl. The essential oils were

injected at a concentration of 10 mg/ml. Helium was the carrier gas (7 psi). The oven temperature was programmed as follows: 40 °C as the initial temperature (maintained for 2 min) to 150 °C in 24 min at 5 °C/min and from 150 °C to a final temperature of 300 °C at 15 °C/min.

A solvent delay of 4 min was selected before analyzing the compounds reaching the MS. Compounds were tentatively identified by MS in SCAN mode, using a mass interval ranging from 35 to 450. Their spectra were compared with those in a MS library (Wiley Registry of Mass Spectral Data) with data found in the literature. Additionaly, in order to identify compounds more precisely, their linear retention indices (RI) were used when possible. Mixtures from *n*-octane to *n*-docosane (Aldrich, Sigma-Aldrich Chemie, Steinheim, Germany) dissolved in *n*-hexane were employed for linear RI calculations.

6.2.6 Optimization methods and statistical processing of experimental data

Using the L9 (3)4 Taguchi design matrix, four variables at three levels each were investigated for the SFE process table 6.1

Tabelul 6.1. The investigated variables and their levels

Factors	1	2	3
Pressure, atm.	100	200	300
Temperature,°C	40	50	60
Static time, minute	60	90	120
Percentage of ethanol, %	0	7	14

SigmaPlot 11.0 Program was employed for statistical analysis of the data with the level of significance set at 95%. One-way analysis of variance (ANOVA) was used to assess statistical differences between extractions. Differences were considered significantly different at a value of $p < 0.05$.

6.3. Results and discussion

Different parameters affecting the extraction yield of the extraction and composition of the extracts were obtained. In fact, pressure, temperature, extraction time and modifier percentage are the most important factors.

Under the analytical conditions tested, the extraction yield ranged between 0.11 and 4.69% (figure 6.6).

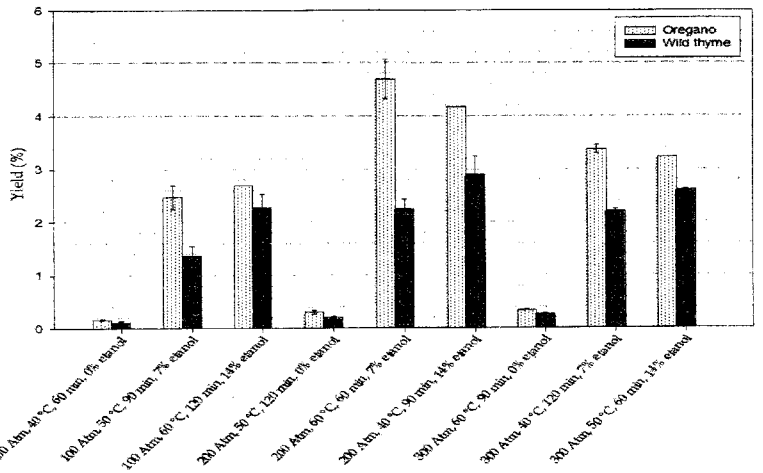


Figure 6.6 Extraction yield

Extraction yield was higher for all extraction conditions when ethanol was used as modifier. Ethanol is used as modifier because CO_2 is a nonpolar compound and is difficult to extract polar compounds such as those found in plants, but this can be solved easily by using a small amount of ethanol.

Compared to other plants, oregano matrix is relatively soft having an extensive influence in the efficiency of the supercritical fluid extraction.

Optimization of the extraction process take into account to obtain essential oils with different phenolic content and antioxidant capacity.

The correlation between changes in the extraction parameters and functional quality of extracts, expressed as total phenolic content and antioxidant capacity are shown in figure 6.7 and 6.8. Experimental datashowed that the most important factor for obtaining essential oils with a higher phenolic content is modifier.

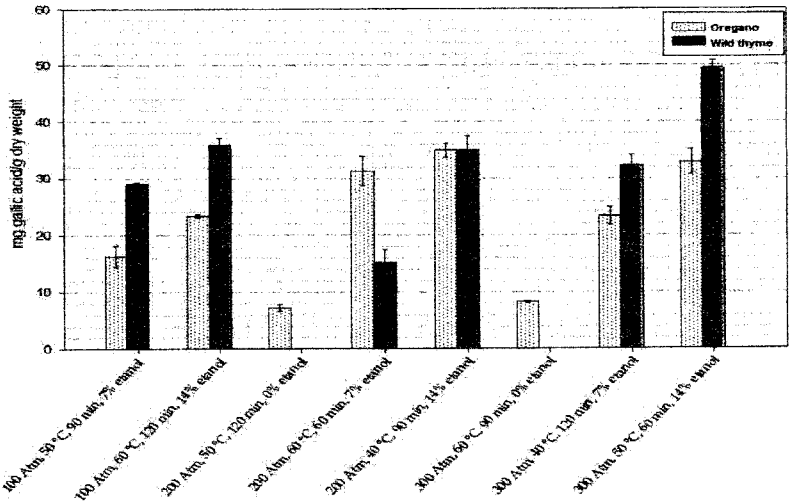


Figure 6.7 Variation of total phenolic content (mg gallic acid/g dry weight), for the supercritical extraction of essential oils from oregano and wild thyme

As shown in figure 6.7 the highest phenolic content was obtained at a pressure of 300 atm, 50 °C, for 60 minutes and 14% ethanol. Moreover, the total phenolic content increased with increasing percentage of ethanol in all the experimental conditions tested.

As shown in figure 6.8, higher antioxidant capacity was obtained when using ethanol as modifier, and wild thyme extracts were most active. The best results were obtained at 300 atm, 50 °C, 60 min and 14 % ethanol.

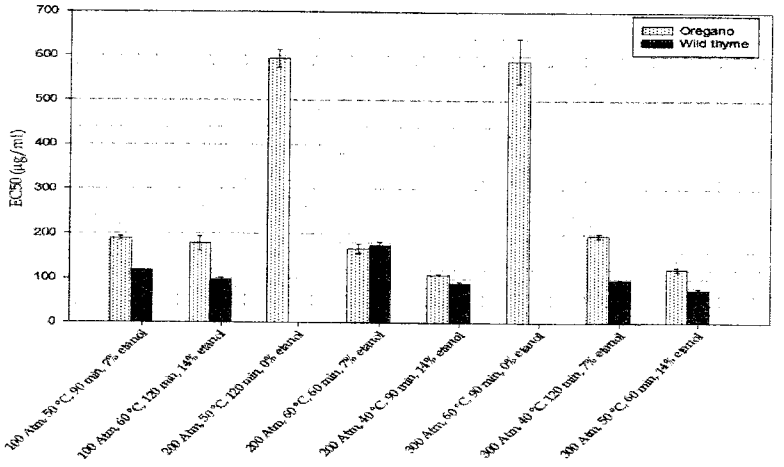


Figure 6.8. Correlation between process parameters and antioxidant capacity (EC_{50}, µg/mL) of essential oils

6.3. Gas chromatography -mass spectrometry (GC-MS) analysis of essential oils

Antioxidant capacity measured by EC_{50} value of oregano essential oils was very low, and this is clearly correlated with its chemical composition.

The chemical composition of essential oils was analyzed using gas chromatography-mass spectrometry. There were identified 18 constituents in the essential oil of oregano that represent 52.22% of the components in the essential oil (figure 6.10, table 6.3).

The main compounds identified in oregano essential oil were caryophyllene oxide (11.23 %), phytol (9.08%), neophytadiene (6,92 %) and trans-sabinene hydrate (5.35%). Also, other important compounds from oregano essential oils with antioxidant capacity were identified: Germacrene D (3,10%), α-terpineol (2,99 %), β-caryophyllene (2,92%), borneol (1,80%), linalool (0,80%), carvacrol (0,45 %).

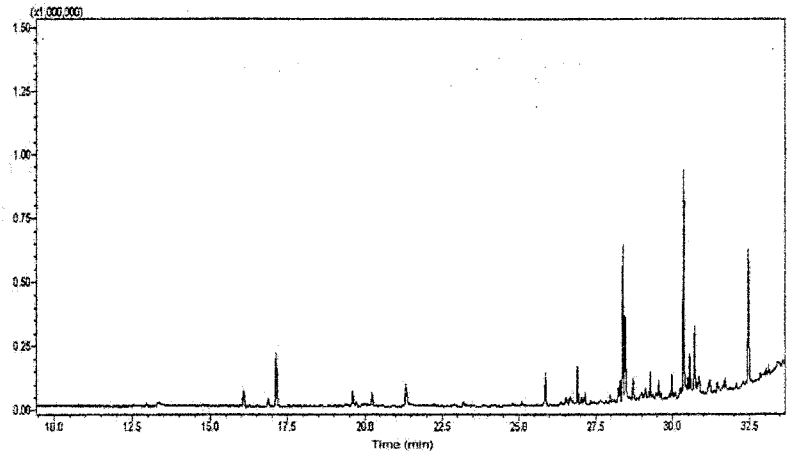


Figure 6.10 Cromatography profile (GC-MS analysis) of oregano essential oils obtained by supercritical fluid extraction (200 atm, 60 °C, 60 min and 7% ethanol

In terms of identifying specific constituents of wild thyme essential oil 25 compounds were identified, representing 74.39 % of the essential oil composition (figure 6.11, table 6.3). The major compounds identified in this oil were thymol (13.99 %) and carvacrol (29.86%).

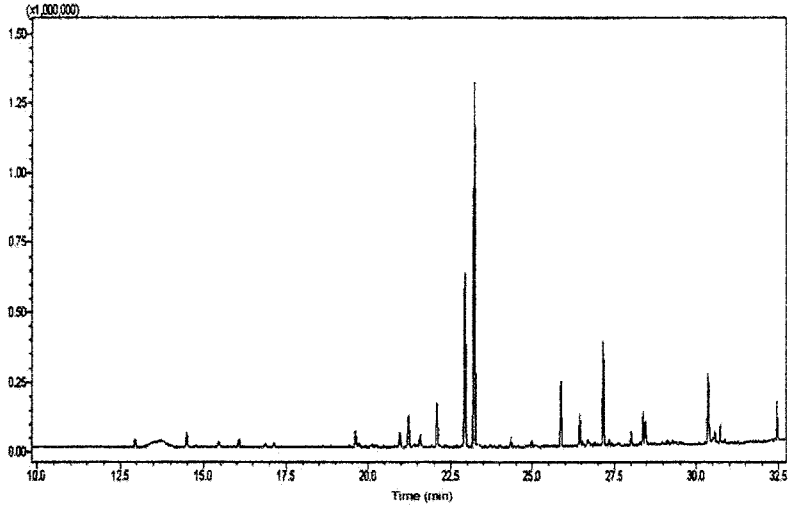


Figure 6.11 Cromatography profile (GC-MS analysis) of oregano essential oils obtained by supercritical fluid extraction (200 atm, 40 °C, 90 min and 14% ethanol

Other major compounds identified in wild thyme essential oi were β-bisabolen (6.08 %), neophytadiene (4.39 %), β-caryophyllene (4.39 %), phytol (2.79 %) caryophyllene oxide (1.99 %) and borneol (1.45 %).

Tabelul 6.3. Bioactive compounds identified by GC-MS in the essential oils of oregano and wild thyme

Nr.	Retention tine, mi	Retention indices, I_k	Identified compounds	Relative content*,%	
				Origanum vulgare	*Thymus serpyllum*
1.	12.93	992.88	1-Octen-3-ol	-	0.79
2.	13.47	1013.02	3-Octanol	0.44	1.60
3.	14.47	1044.03	para-cymene	-	1.37
4.	14.75	1052.71	1,8 cineole	-	0.35
5.	15.45	1074.42	cis-ocimene	-	0.57
6.	16.06	1093.33	cis-sabinene hydrat	1.53	0.69
7.	16.86	1118.14	Linalool	0.80	0.32
8.	17.12	1126.20	trans-sabinen hydrat	5.35	0.44
9.	19.59	1203.22	Borneol	1.80	1.45
10.	19.70	1207.16	Terpinen-4-ol	0.45	0.30
11.	20.09	1221.11	p-cimen-8-ol	-	0.22
12.	20.22	1225.76	α-terpineol	2.99	0.17
13.	22.91	1322.00	Thymol	-	13.99
14.	23.20	1332.38	Carvacrol	0.45	29.86
15.	24.34	1373.17	α-terpinyl acetate	-	0.79
16.	25.08	1399.64	β-burbonene	0.59	0.18
17.	25.86	1451.33	β -caryophyllene	2.92	4.39
18.	26.51	1494.67	α - humulen	1.01	0.39
19.	26.89	1520.00	germacrene D	3.10	0.29
20.	27.03	1529.33	Farnesene	0.69	-
21.	27.14	1536.67	β - bisabolen	1.24	6.08
22.	27.41	1554.67	β-sesquiphelandrene	-	0.19
23.	28.27	1618.46	Spathulenol	1.63	0.25
24.	28.37	1628.72	caryophyllene oxide	11.23	1.99
25.	30.35	1840.13	Neophytadiene	6.92	4.93
26.	32.45	2127.41	Phytol	9.08	2.79

**Extraction conditions*: 200 atm, 60 °C, 60 min and 7% ethanol, for oregano; 200 atm, 40 °C, 90 min and 14 % ethanol, for wild thyme

6.4. Conclusions

It has been demonstrated the utility of supercritical fluid extraction (SFE) using CO_2 as solvent and ethanol as modifier to obtain essential oils.

The highest extraction yields were: 200 atm, 60 °C, 60 min and 7% ethanol for oregano and 200 atm, 40 °C, 90 min and 14 % ethanol for wild thyme.

The addition of ethanol as modifier significantly enhanced the extraction efficiency of polyphenols and antioxidant capacity of essential oils comparing with essential oils obtained with pure CO_2.

The composition of essential oils of oregano and wild thyme was determined by gas chromatography mass spectrometry. Thus, 26 bioactive compounds were identified.These contribute to the total antioxidant capacity of the studied essential oils. The most important are: thymol, carvacrol, germacrene D, 1.8 cineole, linalool, borneol.

The results may suggest that the supercritical essential oils possess compounds with antioxidant activity, and therefore can be used as a natural preservative in food industry.

7. Chemical and functional characterization of bioactive pressurized extracts from aromatic plants

7.2. Materials and methods

Samples and chemicals

Three different plants, belonging to three botanical families which are commonly grown in Romania, were chosen for this study: oregano (*Origanum vulgare*), tarragon (*Artemisia dracunculus*) and wild thyme (*Thymus serpyllum*). The plant samples were obtained from a local herbalist's shop (Galati, Romania) and dried using a traditional method.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity) was obtained from Sigma-Aldrich (Madrid, Spain), ethanol from VWR BDH Prolabo (Madrid, Spain) and methanol from Panreac Quimica (Barcelona, Spain). 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Fluka (Buchs, Switzerland). Folin-Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) whereas antioxidant standards, i.e., gallic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were supplied by Sigma-Aldrich (Steinheim, Germany). CO_2 (N-48) was provided by

Praxair (Madrid, Spain). The water used was Milli-Q Water (Millipore, Billerica, MA, USA). For the UPLC-MS/MS analyses, MS grade ACN and water from LabScan (Dublin, Ireland) were employed.

Probiotic lactic acid cultures, *Lactobacillus acidophilus* and *Lactobacillus casei* were provided from Chr. Hansen, Denmark, as freeze-dried commercial starters. The storage and maintenance of the cultures were carried out according to the recommendation of the manufacturers. Dimethylsulfoxide (DMSO, 99.9 % purity) was obtained from Fluke (Switzerland). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95 % purity) was obtained from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) whereas gallic acid was supplied by Sigma-Aldrich (Steinheim, Germany).

7.2.1. Pressurized liquid extraction (PLE)

PLE extractions of plants were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA). Two different solvents (i.e., water and ethanol) and their mixtures were used in order to obtain extracts with different compositions. Extractions using either 100% water or 100% ethanol were performed at four different extraction temperatures (50, 100, 150, 200 °C). In order to test the influence of the solvent composition, extractions using water/ethanol mixtures were performed at a fixed temperature of 100°C. The extraction time was maintained constant for all the experiments (20 min). An extraction cell heat-up step was carried out for a given time prior to any extraction. The warming-up time changed depending on the extraction temperature (i.e., 5 min when the extraction temperature was 50 and 100°C, 7 min if the extraction temperature was 150°C, and 9 min if the extraction temperature was 200°C). All extractions were done using 11 mL extraction cells, containing 1.5 of sample. When water was used for the extraction, the extraction cell was filled with sand mixture on the top of the sample (2.0 g of sand) to prevent the clogging of the system. Extraction procedure is as follows: (i) sample is loaded into cell, (ii) cell is filled with solvent up to a pressure of 1500 psi (1 psi = 6894.76 Pa), (iii) heat-up time is applied, (iv) static extraction takes place (i.e. 20 min) in which all system valves are closed, (v) cell is rinsed (with 60 % cell volume using

extraction solvent), (vi) solvent is purged from cell with N_2 gas and (vii) depressurization takes place. Between extractions, a rinse of the complete system was made in order to overcome any carry-over.

Once extractions were finished, solvents were removed. For the evaporation of the ethanol, a Rotavapor R-210 (from Buchi Labortechnik AG, Flawil, Switzerland) was used. The water extracts were lyophilized using a freeze-dryer (Labconco Corporation, Missouri, USA).

2.6. LC-MS/MS analyses.

The LC-MS/MS analyses were carried out using an Accela (Thermo Scientific, San Jose, CA) liquid chromatograph equipped with a DAD and an autosampler. The chromatograph was coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole mass spectrometer via an electrospray interface. The analytical conditions employed consisted of a Hypersil C_{18}-AR column (150 mm×4.6 mm, d.p. 3 μm) (Thermo Scientific) using as mobile phases ACN (0.1% formic acid, A) and water (0.1% formic acid, B) eluted according to the following gradient: 0 min, 95% B; 5 min, 95% B; 35 min, 40% B; 55 min, 5% B; 60 min; 5% B; 65 min, 95% B; 70 min, 95% B. The optimum flow rate was 0.4 mL/min while the injection volume was 10 □L. The diode array detector recorded the spectra from 200 to 500 nm.

The MS analyzer was operated under ESI negative mode with the following parameters: Q1 and Q3 resolution of 0.7 Da FWHM; scan width, 0.010 Da; scan time, 0.206 s; spray voltage, 3000 V; sheath gas pressure, 35 psi; auxiliary gas pressure, 5 psi; capillary temperature, 350 °C, skimmer offset (MS/MS experiments), 30 V.

Functional characterization of bioactive pressurized extracts from aromatic plants

- Determination of total phenolic content by Folin-Ciocalteu method
- Determination of antioxidant capacity by DPPH method
- Determination of antioxidant capacity by TEAC method
- Evaluation of antimicrobial activity
- Growth medium for culturing probiotic bacteria
- Viability of probiotic bacteria under simulated gastric juice

7.3. Results and discussion

The particular chemical composition of plants may vary depending of a number of parameters, including geographical-related factors, growing conditions as well as genetic variability. For this reason it is interesting, not only knowing the general chemical composition of a given plant species but also the particular proportions in which these compounds may be present on plants with different geographical origin. With the aim to obtain bioactive compounds from the three studied Romanian plants (i.e., tarragon, wild thyme and oregano), different PLE extraction conditions were tested. The goal of this screening was to use very different extraction conditions in order to have a selected number of extracts of different composition and associated bioactivity. Thus, extracts obtained at the different studied conditions were functionally characterized according to their antioxidant activity and chemically characterized to know their exact composition and to correlate both.

7.3.1. Analysis and optimization of extraction conditions

As mentioned, two different solvents were selected for PLE of Romanian plants, that is, ethanol and water, that cover different polarities. Besides, four different temperatures were also employed for the two solvents (50, 100, 150 and 200ºC), covering the whole instrument's temperature working range. Based on our previous experience with natural matrices (Herrero *et al.* 2010, the pressure was maintained during the whole extraction procedure at 1500 psi and the static extraction time was set at 20 min. This pressure was selected considering that once the extraction pressure is enough to maintain the solvent in the liquid state, its effect is not statistically significant on the outcome of the extraction. Likewise, it has been statistically demonstrated that the influence of the static extraction time is not extremely high (Herrero *et al.*, 2005), and that 20 min is sufficient to ensure the complete extraction of valuable compounds from natural matrices (Plaza *et al.*, 2010a). Moreover, in order to more precisely study the influence of the solvent, different proportions of water and ethanol were combined, namely 25/75, 50/50 and 75/25. To perform these experiments, a medium temperature (100 ºC) was selected.

Figure 7.5 shows the results in terms of extraction yield for the different conditions tested and the three studied plants. As it can be observed, the highest yield was obtained by PLE using water at 200°C for the three plants, being maximum for oregano, reaching more than 60 %, whereas the lowest yields were obtained using ethanol as solvent at 50 °C (particularly the yield obtained for wild thyme, 3.2 %).

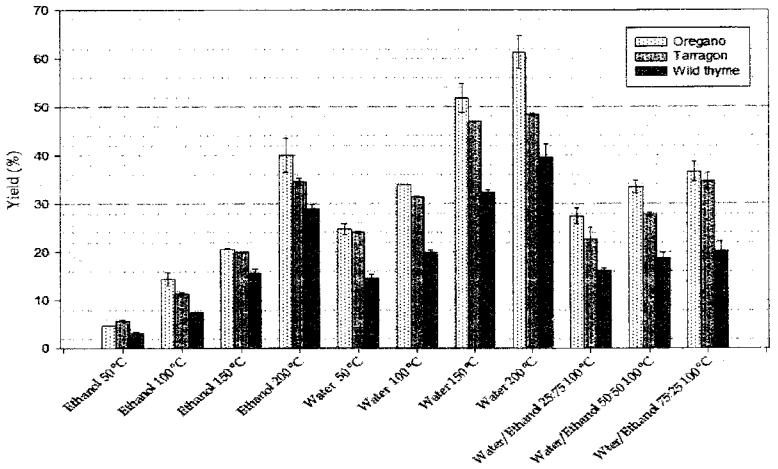


Figure 7.5 Extraction yield (%) produced after the PLE extraction of the three studied plants at the indicated conditions

Considering the different extraction temperatures tested, the extraction yield was higher when increasing the temperature, independently of the solvent employed. For the same temperature, in all cases significantly higher yields were obtained with water compared to those with ethanol. In agreement with this observation, when the extraction temperature was maintained at 100°C and the solvent composition was changed, the extraction yield increased when higher proportions of water were employed. Interestingly, similar yields were obtained with 100 % water and a mixture water/ethanol 75:25. These results suggest that most of the compounds present on these plants had a relatively high polarity, and therefore, were preferentially extracted with ethanol and, above all, with water. The increase of

extraction yield with the temperature corresponded to a typical increment of the mass transfer as a result of the application of higher temperature as well as to a decrease on the solvent viscosity which helps the solvent to penetrate the matrix.

7.3.2 Evaluation of total phenolic content and antioxidant capacity of plant extracts

The next step consisted on the functional analysis of the extracts: assays such as Folin-Ciocalteau, DPPH and TEAC were used to assess both, the total phenols and the antioxidant activity of the extracts obtained under the screened conditions; data is presented in figure 7.6, 7.7, 7.9. In terms of total phenols, it can be seen that oregano was, by far, the richest plant in terms of total phenols followed by wild thyme and tarragon; this behavior was maintained in all the PLE conditions tested.

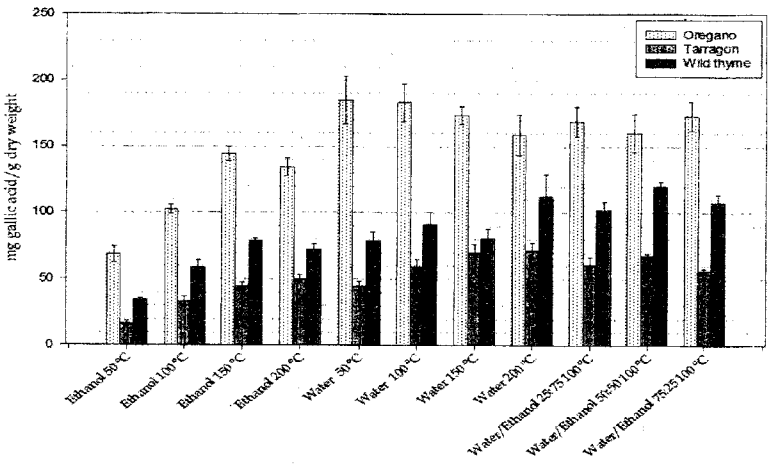


Figure 7.6 Total phenolic content (mg gallic acid/g dry weight) of bioactive pressurized extracts

On the other hand, the highest amount of total phenols was obtained with pressurized water for all the studied plants. However, the behavior of the different plants as a response of the increase of temperature was different. Whereas oregano extracts presented a maximum at 100 °C, 200 °C was the most efficient temperature for phenol's extraction in tarragon and wild thyme. In both cases, a higher extraction

temperature meant a higher amount of total phenols extracted for the two tested solvents. When keeping the extraction temperature constant at 100 °C, it could be observed how the maximum amount of total phenols was attained using a mixture of ethanol/water 50:50 for tarragon and wild thyme, whereas for oregano 100% water provided with better results. Nevertheless, the amount of total phenols obtained from oregano with the three solvent mixtures water/ethanol were not statistically different ($p > 0.05$). Nonetheless, looking at the results as a whole, it can be affirmed that the three plants, particularly oregano, were rich on phenols, and thus, had the potential for providing with active antioxidant extracts.

Two methods to assess the antioxidant capacity of the extracts were selected, namely DPPH radical scavenging assay and TEAC (Trolox equivalents antioxidant capacity) assay. The use of two different antioxidant capacity methods may provide a deeper insight on the chemical constituents present on the extracts as well as their different activity against different radicals. The results collected using these procedures are summarized in figure 7.7.

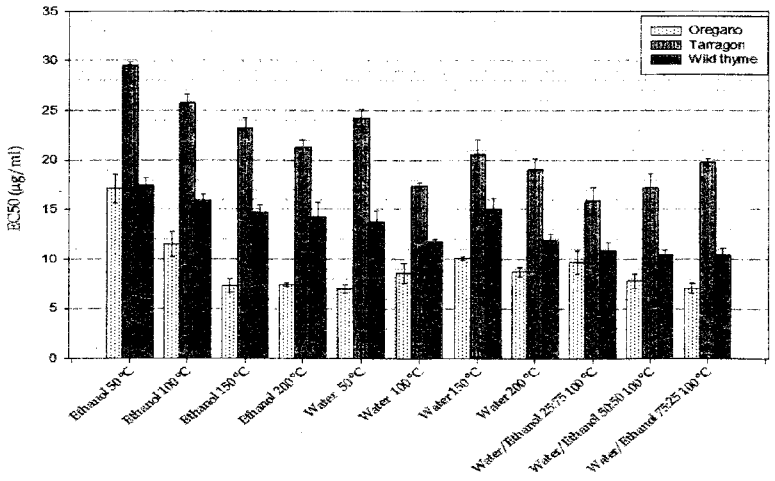


Figure 7.7 Antioxidant capacity expressed as EC_{50} (µg/ml) of bioactive pressurized extracts

It is important to consider that the results from the DPPH method were expressed as EC_{50} and therefore, the lowest the value, the highest the antioxidant capacity. As can be seen, the best results in terms of EC_{50} were obtained for oregano. As a general trend for the three plants, an increase of extraction temperature using ethanol provided a higher antioxidant capacity, although values obtained for extractions at 150 and 200 °C (using ethanol) were not statistically different ($p > 0.05$). In the case of the PLE extractions using water, an increase in the antioxidant capacity was generally observed when the temperature was raised from 50 to 100 °C, then decreased and finally increased again at 200 °C. This behavior can be explained by a improved recovery of antioxidant compounds at temperatures up to 100 °C and a subsequent degradation at higher temperatures. The improvement of antioxidant activity at 200 °C, can be due to other phenomena that can occur at very high temperatures using water as extraction solvent, such as the neoformation of antioxidant compounds derived from Maillard reaction, among others (Plaza *et al.*, 2010b). These phenomena have been demonstrated to occur in natural matrices, containing reducing sugars and aminoacids, to some extent, therefore contributing to the total antioxidant capacity of the extracts compared to those obtained at 150 °C.

Combining the information regarding the antioxidant capacity in terms of EC_{50} and total phenols' content, it can be observed how there is a clear correlation between the two measurements (figure 7.8) indicating that the samples with a higher content on total phenols were, in general, also the most active in terms of antioxidant capacity.

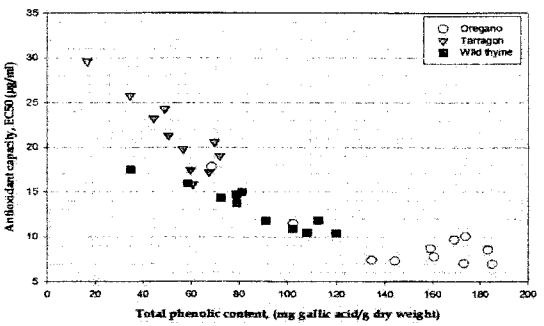


Figure 7.8. Correlation between the amount of total phenols determined on the plant extracts and their corresponding activity measured using the DPPH

This behavior has previously been suggested for different natural matrices including plants, algae and vegetables (Mendiola *et al.*, 2009). As it can be observed in this figure, only in the case of oregano, some extracts possessed the same antioxidant activity or even higher than other which, however, were richer on total phenols. In this case, as mentioned, partial degradation of total phenols could occur when extracting with water at the highest temperature while, at the same time, new antioxidants might be forming at these conditions.

As for the results of TEAC assay (figure 7.9), extracts followed the same trend previously mentioned for EC_{50} values but, in this case, higher values corresponded to higher antioxidant capacity. Both methods measured the ability of an antioxidant to transfer an electron and scavenge a radical (DPPH or ABTS), thus, considering similar mechanisms, an equivalent behavior is expected.

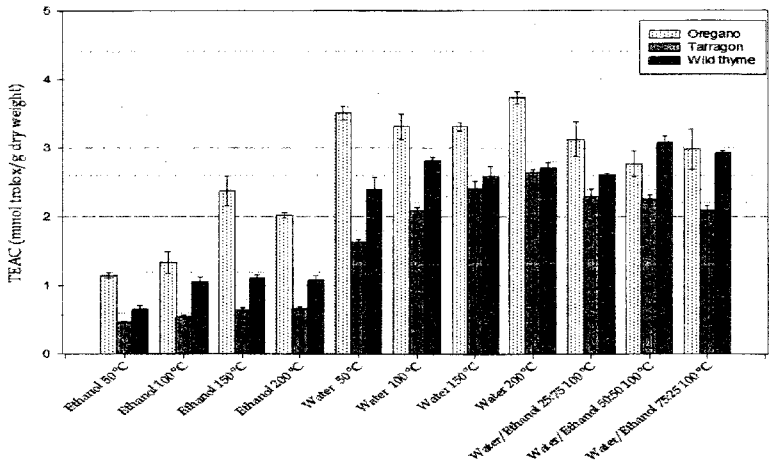


Figure 7.9 Antioxidant capacity expressed as TEAC (mmol trolox/g dry weight) of bioactive pressurized extracts

7.3.4. In vitro stimulatory effect of oregano extract on multiplication and viability maintenance of probiotic bacteria Lactobacillus acidophilus and Lactobacillus casei

Starting from an inoculums of 8.57 log CFU/mL for *Lactobacillus acidophilus* and 6.32 log CFU/mL for *Lactobacillus casei*, after 4 hours of cultivation, at 37 °C, in MRS broth and MRS broth supplemented with 20 mg/mL *Origanum vulgare* L.

extract the count clearly showed that vegetal extract rich in phenolic compounds stimulate the growth of the probiotic bacteria.

The figure 7.13 shows that DMSO solution (control 2) inhibits the cell growth in probiotics compared to the culture in MRS broth (control 1) (from 8.71 log to 8.69 log CFU/mL) but the cultivation of *Lactobacillus acidophilus* strain on MRS broth with *Origanum vulgare* L. extract dissolved in DMSO led to a growth compared to control from 8.71 log to 8.94 log CFU/mL.

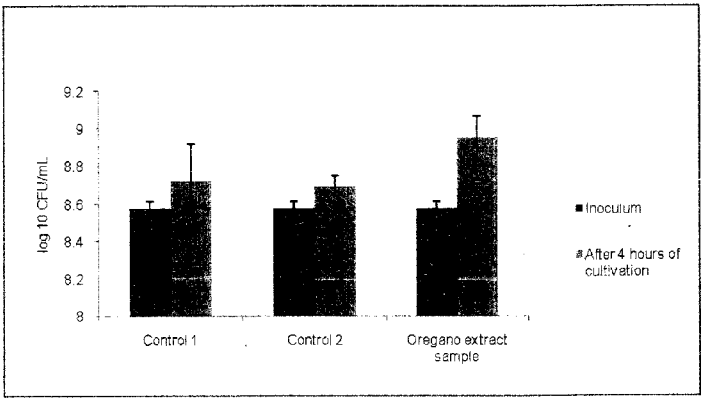


Figure 7.13. The influence of the *Origanum vulgare* L. extract on the growth of the *Lactobacillus acidophilus*

As can be observed control 2 containing 20 % DMSO solution, the solvent used to dissolve oregano extract, inhibits the probiotic cells growth by 15 % compared to control 1. Nevertheless, the cultivation of *Lactobacillus acidophilus* bacteria on MRS broth with *Oregano vulgare* L. extract seemed to be more active leading to a growth by 163 % compared to control 1. These data could indicate that the polyphenols compounds are responsible for the stimulatory role on probiotic cells growth as previously described by Hervert-Hernández et al. [8].

Figure 7.14 shows the results after 4 hours cultivation at 37 °C of *Lactobacillus casei*. As in the experiment above presented, it can be seen that control 2 slightly inhibits the growth of the probiotic *Lactobacillus casei*, but on the other hand it can be observed that sample with *Origanum vulgare* L. extract stimulated the growth of the probiotic compared to control 1, from 6.45 log to 6.69 log CFU /mL. Compared to

control 1 the oregano extract sample has a 200 % growth of the probiotic cell number.

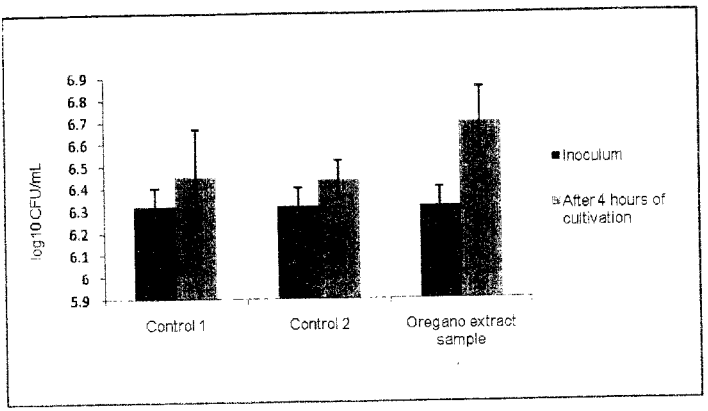


Figure 7.14. The effect of the vegetal extract addition on the growth of the *Lactobacillus casei*

The cultivation of the two probiotic bacteria species (*Lactobacillus acidophilus* and *Lactobacillus casei*) that was carried out in MRS broth, led to the conclusion that the stimulation of the probiotic bacteria growth by the *Origanum vulgare* L. extract is significant both in the case where *Lactobacillus acidophilus* was used as cultivation strain and in the case where *Lactobacillus casei* was used.

7.3.5. Influence of oregano extract on improving stability of probiotic bacteria under simulated *in vivo* conditions

The presence of biologic compounds of the vegetal extracts could be reasons for the successful survival of bacteria during the simulated gastric juice tests.

The viability of *Lactobacillus acidophilus* and *Lactobacillus casei* cells during simulated gastric digestion in the presence of *Origanum vulgare* L. extract is presented in figures 3 and 4.

The cell number of the *Lactobacillus acidophilus* in sample was higher than control 1, from 7.75 to 7.86 CFU/mL, after 30 minutes. After 60 minutes of incubation in the same experimental conditions, the cell number of the sample was also higher than the control 1, from 7.67 to 7.86 CFU/mL. From these results, after 30 and 60

minutes of incubation respectively, a reduction of the [illegible] *acidophilus* was observed for all the three variants (figure 7.15

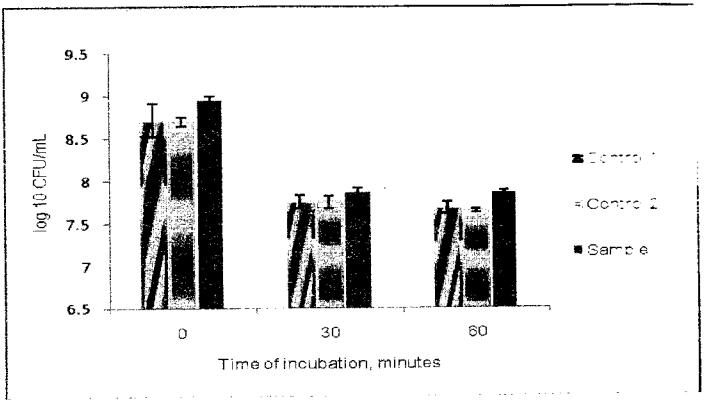


Figure 7.15. The influence of the *Origanum vulgare* L. extract of the viability *Lactobacillus acidophillus* in simulated gastric juice

Comparing the cell number of the sample with that of the control 1, at 30 minutes, it is observed that these are 130 % higher than control 1. After 60 minutes of incubation in the same experimental conditions, the cell number in the sample is 151 % higher than control 1.

Comparing the cell viability at 30 minutes, 60 minutes and 90 minutes a stimulation effect is observed on *Lactobacillus casei* in presence of the vegetal polyphenols (sample) comparing with the controls. Thus, the growth of the cell number compared to the control 1 is from 3.43 log to 4.3 log CFU/mL after 30 minutes incubation in gastric juice, from 1.95 log to 1.97 log CFU/mL after 60 minutes and from 1.15 log to 1.6 log CFU/mL after 90 minutes (figure 7.16).

Comparing the cell viability at 30 minutes, 90 minutes and 120 minutes a viability stimulation effect is observed on *Lactobacillus casei* in sample. Growth of probiotic bacteria *Lactobacillus casei* was affected by phenolic compounds compared to control 1 with following trend: 105.4 % at 30 minutes incubation time in gastric juice; 126 % after 90 minutes; 201 % after 120 minutes.

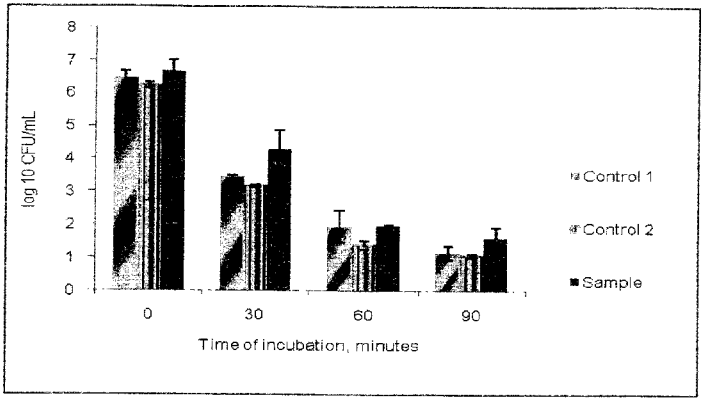


Figure 7.16. The *Origanum vulgare* L. extract influence of the viability *Lactobacillus casei* in simulated gastric juice

In the presence of *Origanum vulgare* L. extract, both strains showed an enhanced survival in comparison with the negative controls samples.

The high recovery rate of the total probiotic bacterial population maintained in presence of the *Origanum vulgare* L. extract could be explained by the positive and protective action of biologic active compounds upon cells physiology and resistance.

7.3.6. Chemical composition of bioactive pressurized extracts from aromatic plants

An LC-MS method was adapted to characterize the obtained PLE extracts from the three studied plants. A quite slow gradient was employed, not chasing a fast analysis but a higher resolution of the complex profiles of the different extracts. In anexe 1, the chromatograms corresponding to the extracts obtained by PLE using water and ethanol as solvents at 200°C from the three studied Romanian plants are shown. As it can be appreciated, even if the six profiles were very different, a good separation of the compounds was achieved. Identification of compounds was attempted combining the information provided by the DAD and by the MS detector together with retention times and information available on the literature. Particularly useful was the combination of UV-Vis and MS spectra together with data regarding the fragmentation of the main ions detected.

Using this approach, different compounds could be identified or tentatively assigned on the different samples. Identification of compounds is shown in Table 2, together with the data collected using the two detectors (DAD and MS) in series. Besides, the plant in which each compound was found is also indicated.

Oregano PLE extracts

As it can be observed in Annex 1 A and B, together with the information given in Table 2, the profile obtained when using water as extraction solvent was different than with ethanol. As expected, the main differences were observed for the less polar compounds that were preferably extracted using ethanol. When a mixture ethanol/water was employed, results were similar to those obtained only using water; these results are in agreement with those on total phenols that, for mixtures, were closer to the values obtained with water at the same temperature.

The main phenolic antioxidant present on the extracts obtained with water was rosmarinic acid (peak 21); Other important compounds in these extracts were luteolin-7-*O*-glucuronide (peak 15) as well as luteolin (peak 22) and different phenolic acids including syringic (peak 1), protocatechuic (peak 2), homovanillic (peak 3), chlorogenic (peak 6), hydroxybenzoic (peak 7) and caffeic (peak 10) acids. For the characterization of the phenolic acids, typical UV-Vis spectra as well as their corresponding $[M-H]^-$ ions and common fragments were found. On the other hand, the peak corresponding to luteolin-7-*O*-glucuronide presented a molecular ion ($[M-H]^-$) at m/z 461.1. Besides, the UV-Vis spectrum matched with that corresponding to luteolin, characterized by a maximum absorbance at 340 nm. Moreover, the detection of the fragment corresponding to luteolin (m/z 285) corroborated its identification. Higher amount of phenolic compounds were extracted when using water at 100 °C compared to the extraction at 200 °C (see Table 1). Nevertheless, qualitatively, the main difference among these two extracts was the lack of extraction of less polar antioxidants, mainly luteolin at the lower temperature. Also at 100 °C (chromatogram not shown), apigenin-7-*O*-glucuronide could be tentatively identified since its molecular ion, as well as the fragment corresponding to apigenin, were detected, together with the match of its UV-Vis spectrum. This compound was not recovered when using water at 200°C, probably because of too higher temperatures led to its degradation.

Table 7.1. Compounds identified in the PLE extracts analyzed by LC-MS.

ID	Retention time (min)	Compounds identified	UV-Vis maxima (nm)	$[M-H]^-$	Main fragments detected	Plant in which was detected
1	12.7	Syringic acid	280	197.1	179, 135	O, Wt
2	14.5	Protocatechuic acid	260, 293	153.1	108	O
3	15.6	Homovanillic acid	277	181.2	167, 137	O
4	15.7	3-Caffeoylquinic acid	297, 325	353.2	191, 179	T
5	17.7	Vanillic acid	277	167.2		Wt
6	17.8	Chlorogenic acid	300, 326	353.3	191	O, T, Wt
7	17.9	Hydroxybenzoic acid	282, 312s	137.1		O, Wt
8	18.0	4-caffeoylquinic acid	299, 326	353.2	191, 173	T
9	18.2	*p*-Coumaric acid	286	163.1	137	Wt
10	19.3	Caffeic acid	291,323	179.2	135	O, Wt
11	19.4	Caftaric acid	298,326	311.2	179	T
12	20.9	Luteolin-7-*O*-glucoside	265, 340	447.2	285	Wt
13	21.9	Rosmarinic acid isomer	291, 329	359.1	161	O
14	22.2	Protocatechuic glucoside	264, 287s	421.1	153	O
15	22.4	Luteolin-7-*O*-glucuronide	265, 347	461.1	285	O, Wt
16	23.0	Eriodictyol-7-*O*-glucuronide	283, 329s	463.2	287, 175	Wt
17	23.3	Dicaffeoylquinic acid	300, 325	515.2	353, 191, 173	T
18	24.2	Dicaffeoylquinic acid	299, 328	515.2	353, 191, 173	T
19	24.3	Apigenin-7-*O*-glucuronide	267, 334	445.2	269	Wt
20	24.5	Dicaffeoylquinic acid	298, 327	515.3	353	T
21	25.0	Rosmarinic acid	291, 329	359.2	161	O, Wt
22	28.5	Luteolin	265, 347	285.2		O, Wt
23	29.1	Eriodictyol	287	287.2	151	Wt
24	29.9	Caffeic acid ethyl ester	299, 323	207.2	179, 161, 135	O, T, Wt
25	30.0	Naringenin	284, 330s	271.2		O
26	31.2	Apigenin	332	269.1		O, Wt
27	31.7	Cirsimaritin	338	313.2		Wt
28	32.0	Prenylnaringenin	261, 321s	339.8	271	Wt
29	32.3	Isorhamnetin	286s, 360	315.2		T
30	32.4	Quercetin	287, 345s	301.2		T

s, shoulder; O, oregano; T, tarragon; Wt, wild thyme

Concerning the ethanol extracts, their chromatographic profiles were very similar, although a higher amount of phenolics could be obtained at the highest temperature (Table 7.1). In these extracts, rosmarinic acid (peak 21) was also among the main components present, although also luteolin (peak 22) and caffeic acid ethyl ester (peak 24) could be also extracted in high amounts. Regarding this latter compound, identification was based on the combination of the typical UV-Vis spectra of an hydroxycinnamic acid, with absorption maxima at 299 and 323 nm, together with a molecular weight ([M-H]-) of 207.2. This information suggested the presence of a hydroxycinnamic acid derivative. Moreover, the fragmentation of this base peak provided with fragments corresponding to m/z 179, 161 and 135, typical of caffeic acid. Thus, combining all this information, this peak could be tentatively assigned to caffeic acid ethyl ester, as it is shown in Figure 7.17

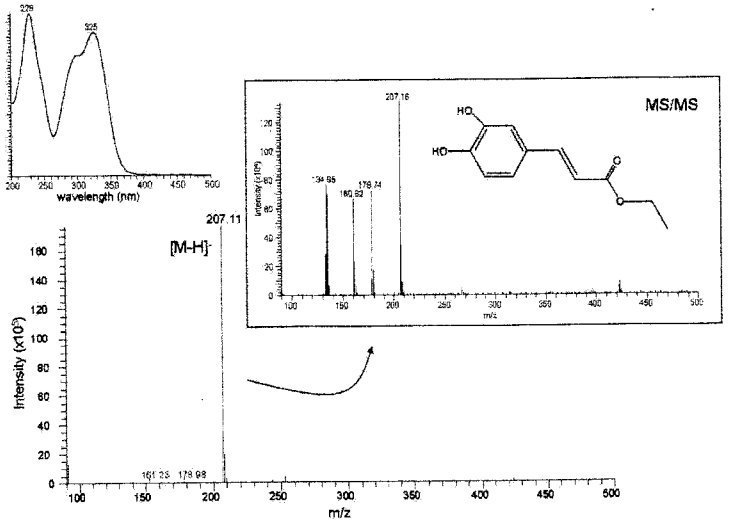


Figure 7.17 UV-Vis and MS spectrum of caffeic acid ethyl ester (m/z 207.1, peak 23), as well as its fragmentation pattern and proposed chemical structure

In general, a total of 14 different compounds could be tentatively identified in the Romanian oregano extracts. Besides, as it can be observed in Annex 1, other important peaks in the chromatograms could not be successfully assigned; information regarding their UV-Vis maxima, molecular ion and main fragments

detected is shown in Table 7.2. For instance, peak f showed UV-Vis and MS spectra that may indicate the presence of dyhydroxykaempferol. The retention time of this peak could also confirm this tentative assignment. However, due to the absence of a clear fragment at m/z 259, this peak could not be successfully assigned.

Table 7.2. UV-Vis and MS data of the main peaks detected in the PLE extracts analyzed by LC-MS which identity could not be confirmed.

ID	Retention time (min)	UV-Vis maxima (nm)	$[M-H]^-$	Main fragments detected	Plant in which was detected
a	19.9	277	329.2	167	O
b	21.0	281	393.2	231, 123	O
c	21.1	283, 335	639.2	609, 451	T
d	21.5	294, 319	481.3	355, 193	T
e	21.7	263, 283s, 295s	437.2	153	O
f	24.0	283, 325	287.2	243, 121	O
g	33.2	276, 310	257.2		T
h	38.6	288, 331s	285.2		T
i	39.8	266	207.2		T
j	40.3	276, 310	271.3		T

s, shoulder; O, oregano; T, tarragon; Wt, wild thyme

Wild Thyme PLE extracts

The chemical characterization of the wild thyme extracts by LC-MS revealed that those obtained with water and with water/ethanol mixtures did not differ significantly from a qualitative point of view; this is in agreement with the total phenols observed for both, water and water/ethanol extracts (Table 7.1), as mentioned previously for oregano extracts. However, those extracts obtained with ethanol possessed a different composition. As it can be clearly observed in Annex 1 E and F, less polar compounds dominated in the ethanol extract chromatogram whereas more polar compounds were extracted with water. Among them, rosmarinic acid (peak 21) was the main compound in the wild thyme water extracts. Besides,

other polar phenolic acids were also detected, notably, syringic (peak 1), vanillic (peak 5), chlorogenic (peak 6), *p*-coumaric (peak 9) and caffeic (peak 10) acids. All these phenolic acids are an important influence on the total antioxidant capacity shown by these extracts. Moreover, other flavonoids such as luteolin-glucoside, luteolin-glucuronide, eriodictyol-glucuronide, apigenin-7-*O*-glucuronide (compounds 12, 15, 16 and 19, respectively) could be identified together with the aglycones luteolin, eriodictyol and apigenin (peaks 22, 23 and 26). The different glucuronides were clearly assigned based on the detection of their molecular ions as well as the fragments corresponding to their aglycones. Data on UV-Vis spectra was used to confirm the identification. This combination allowed, for instance, the correct assignment of the ion with m/z 463, as it can be appreciated in Figure 7.18.

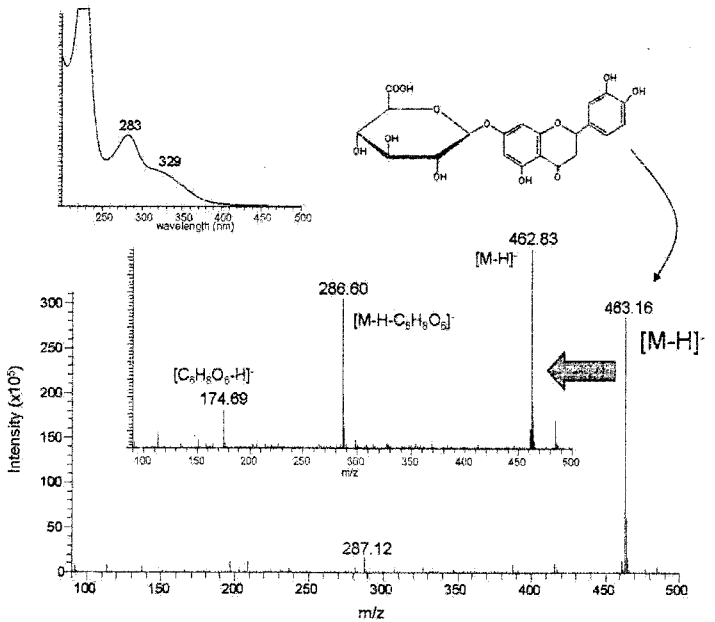


Figure 7.18 UV-Vis and MS spectra of eriodictyol-glucuronide (peak 15) and fragmentation pattern and chemical structure proposed for this assignment

Considering that this compound should be a flavonoid, in agreement with its retention time and UV-Vis spectrum, the detection of a fragment derived from the

main peak of m/z 287 permitted the assignment of this compound as an eriodictyol derivative instead of other with similar molecular weight, such as isoquercetin. Besides, a fragment of m/z 175, typical from the glucuronide moiety, was detected, supporting also this assignment. Although the possibility of assigning positional isomers could be theoretically achieved by using MS, under the conditions employed in the present research, these glycosilated flavonoids could not be unambiguously characterized. Nevertheless, their more frequent forms, containing a 7-*O*-linkage were assumed.

On the other hand, in the wild thyme ethanol extracts, rosmarinic acid was not the main identified compound, although its presence could also be confirmed. Instead, important peaks appeared later on the chromatogram, corresponding to luteolin (peak 22), apigenin (peak 26) and in less extent, eriodictyol (peak 23), cirsimaritin (peak 27) and prenylnaringenin (peak 28). This last compound was assigned thanks to the detection of a base peak at m/z 339.8 ([M-H]$^-$) together with a typical fragment of m/z 271 corresponding to the loss of the prenyl moiety. Nevertheless, the main compound in these chromatograms (see Figure 3) was again caffeic acid ethyl ester (peak 24). This compound appeared also in the ethanol extracts of the other two Romanian plants studied.

In conclusion, a total of 17 different phenolic compounds could be tentatively identified in the wild thyme PLE extracts, which may probably have a strong influence on the total antioxidant activity observed.

Tarragon PLE extracts

As it can be observed in Annex 1 C and D, the profiles obtained for the extracts obtained with water and ethanol at 200°C from tarragon were qualitatively quite similar, although, in general, water extracts possessed higher amount of phenols than their corresponding counterparts obtained with ethanol (Table 7.1). In fact, the same compounds could be basically identified in both extracts. Nevertheless, the water extracts were mainly characterized by the presence of caffeoylquinic (peaks 4, 6 and 8) and dicaffeoylquinic (peaks 17, 18 and 20) acids whereas in the ethanol extracts the major compounds were found at the end of the chromatogram,

corresponding to less polar compounds (e.g., peaks h, i, j). Besides, the same hydroxycinnamic derivative compound also found in oregano, tentatively identified as caffeic acid ethyl ester (peak 24), was the main peak in these extracts. On the other hand, in water extracts, these compounds were found in less amount or not found at all (e.g. compound 24). These acids possess a particular UV-Vis spectrum with absorption maxima at 300 and 325 nm, which detection was used in the present work as a first hint for a possible identification. Next, the information provided by the MS detector was studied. Several of these compounds presented molecular ions ($[M-H]^-$) corresponding to m/z 353 (i.e., peaks 4, 6 and 8, respectively). Among them, the main peak (peak 6) provided a fragment of m/z 191, and was tentatively assigned to chlorogenic acid (Ma *et al.*, 2008). Besides, it is widely known that chlorogenic acid is the principal caffeoylquinic acid in tarragon. On the other hand, compounds 4 and 8 gave fragments of m/z 179 and 173, respectively. According to this latter fragment, typical from the 4-acyl groups, peak 8 was tentatively identified as 4-caffeoylquinic acid, whereas the finding of the fragment m/z 179 in peak 4 suggested that this compound could be 3-caffeoylquinic acid. Besides, three other peaks, eluting later on the chromatogram, presented also the typical UV-Vis spectrum of caffeoylquinic acids. For these compounds (compounds 17, 18 and 20), MS base peaks ($[M-H]^-$) of m/z 515 were detected as well as fragments of m/z 353, thus clearly indicating the presence of dicaffeoylquinic acids. Although these compounds were not fully characterized, the occurrence of fragments at m/z 173 in peaks 17 and 18 indicated the presence of 4-acyl dicaffeoylquinic acids. Examples of the assignment process as well as the structures proposed for compounds 6 and 17 are shown in Figure 5. Besides these compounds, caftaric acid (peak 11) as well as caffeic acid ethyl ester (peak 24) and other flavonoids (isorhamnetin and quercetin, peaks 29 and 30, respectively) were identified in the tarragon extracts.

Other important peaks that could not be completely identified (peaks g, h, i and j, see Annex 1 (C and D) were also detected in the extracts produced using both solvents, although they were in higher extent in the ethanol extracts. Characteristics of these non-identified peaks are shown in Table 7.2.

7.4. Conclusions

The applicability of PLE as an advanced environmentally friendly extraction technique for the extraction and characterization of native Romanian plants such as oregano, tarragon and wild thyme, has been demonstrated.

Different combinations solvents-temperatures were screened to obtain extracts with important bioactivities; extraction yields, antioxidant capacity and chromatographic profiles were studied to obtain a complete picture of the process.

Results showed that higher yields were obtained with water at very high temperatures (200°C), reaching values around 62% when using oregano as raw material. Besides, the higher antioxidant capacity was obtained using water at 50-100°C, being oregano the most active.

Data suggested a direct correlation between the amount of total phenols and the antioxidant activity measured using DPPH radical scavenging protocol.

The growth of both probiotic strains can be stimulated in media with added *Origanum vulgare* L. extract, obtained by pressurized liquid extraction.

The resistance of tested strains by incubation in simulated gastric juice is also positively influenced by adding 20 mg/mL *Origanum vulgare* L. extract after 30 minutes of incubation. After 60 and 90 minutes respectively the viability drastically decreases, but the protective effect of the extract is positive compared with the control.

To improve the positive effects, an optimization of the extract concentration is necessary for the next steps of the study. *Origanum vulgare* L. extract acts as a food vector by sustaining adequate populations of viable bacteria, and improves their survival in gastric digestion.

The development of new probiotic foods should consider not only the intrinsic characteristics of effective bacterial strains but also the ability of the food matrix to protect the bacterial cells all the way through the gastrointestinal tract.

Besides, the use of an LC-MS/MS method allowed the characterization of the phenolic compounds on PLE extracts. Thirty different compounds could be tentatively assigned by using this method, some of them described for the first time in these plants. Oregano extracts were mainly characterized by the presence of phenolic acids, mainly rosmarinic and caffeic ethyl ester acids. Extracts from

tarragon were particularly rich on caffeoyl and dicaffeoylquinic acids, as well as on other flavonoids, whereas wild thyme presented the most complex chemical profile including phenolic acids and different glycosilated flavonoids and aglycons. To the best of our knowledge, the possibility of obtaining such compounds from these species through the application of PLE-*in-vitro* antioxidant assays-LC-MS/MS is shown for the first time.

8. Studies of solubility, stability and delivery of bioactive compounds from aromatic plants

8.2 Materials and methods

For this study were used pressurized extracts of oregano and wild thyme obtained by extraction with water at a temperature of 100 °C (according with the extraction method described at Chapter 7). Ultrapure water was obtained using a water purification equipment Smart2 Pure TKA.

The reagents: 1,1 diphenyl 2-picrylhydrazyl (DPPH) (95 % purity), Folin-Ciocalteu and sodium carbonate (Na_2CO_3) and antioxidant standards, gallic acid and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Steinheim, Germany).

Vegetable oils used for testing the solubility:

- Cumin seed oil
- Peanut oil
- Flaxseed oil
- Almond oil
- Black pepper oil
- Pumpkin seed oil
- Corn germ oil
- Nut oil
- Lemon oil

The surfactant Tween 40, Tween 80 were purchased from Sigma Aldrich, and SPAN 80 was purchased from Fluka (Switzerland). Linoleic acid was purchased from BDH Chemicals Ltd (Poole, England). Synthetic antioxidants butylated hydroxyanisole

(BHA) and butylated hydroxytoluene (BHT) were supplied by KUK (Bucharest, Romania).

Dimethyl sulfoxide (DMSO), 99.9 % purity was purchased from Fluka (Switzerland).

8.2.3 Microemulsions containing extracts from aromatic plants

Microemulsions were formed by adding the following components: nut oil, linoleic acid, glycerol, ethanol, Span 80, Tween 20, Tween 80. The ratio of microemulsion components was changed in order to obtain single-phase microemulsions:

- 50% nut oil + 30% Tween 20 + 20% ethanol
- 50% nut oil + 30% Tween 20 + 20% glycerol
- 50% nut oil + 20% Tween 20 + 30% ethanol
- 50% nut oil + 20% Tween 20 + 20% glycerol
- 50% nut oil + 10% Tween 20 + 40% ethanol
- 50% nut oil + 10% Tween 20 + 40% glycerol
- 40% nut oil + 10% Tween 20 + 50% ethanol
- 40% nut oil + 10% Tween 20 + 50% glycerol
- 50% nut oil + 30% SPAN 80+ 20% ethanol
- 50% nut oil + 30% SPAN 80+ 20% glycerol
- 50% nut oil + 20% SPAN 80+ 30% ethanol
- 50% nut oil + 20% SPAN 80 + 20% glycerol
- 50% nut oil + 10% SPAN 80 + 40% ethanol
- 50% nut oil + 10% SPAN 80 + 40% glycerol
- 40% nut oil + 10% SPAN 80 + 50% ethanol
- 40% nut oil + 10% SPAN 80 + 50% glycerol
- 60 % linoleic acid + 5% Tween 80 + 35% ethanol
- 66,6% linoleic acid + 6,6% Tween 80 + 26,6% ethanol
- 64,5% linoleic acid + 3,22% Tween 80 + 32,22% ethanol

The extracts of oregano and wild thyme were solubilised in a microemulsion (60 % linoleic acid + 5% Tween 80 + 35% ethanol) and then were ultrasonicated at 300 Hz, for 3 min using an equipment SONOPULS.

The phase diagrams facilitate the calculation of a mixture of components, respectively the concentration areas corresponding to the state of microemulsion.

According to conductivity measurements, microemulsions investigated are two types A/U and U/A.

In figure 8.2 are represented the diagrams of two systems:

a) nut oil which in solubilised thyme extract, ethanol, which acts as cosolvent and SPAN 80, which acts as a surfactant
b) linoleic acid which is solubilised wild thyme extract, ethanol, which acts as cosolvent and SPAN 80, which acts as a surfactant

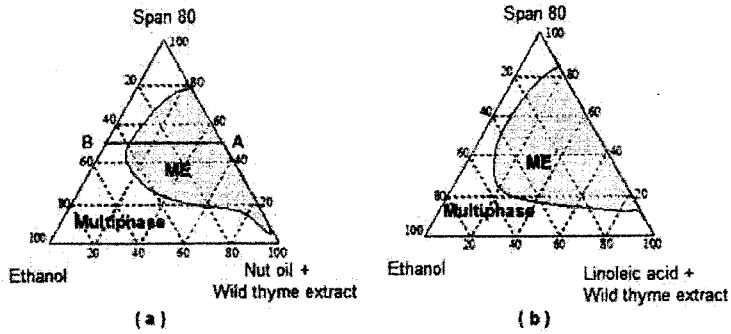


Figure 8.2 Pseudo-ternary diagrams of microemulsions systems

a) ethanol/nut oil – wild thyme extract/Span 80
b) ethanol/linoleic acid- wild thyme extract/Span 80

ME-microemulsion

8.3 Results and discussion

8.3.1. Stability analysis of wild thyme extract under different storage conditions

Since de final objective of this study is the addition of plant extract in a food product to improve the antioxidant capacity was studied the stability of these extracts at different temperatures and different pH values.

As can be obverved in figures 8.3, 8.4 and 8.5, the polyphenols compounds are stable at pH 5 and loss the effiency at the pH 9. Thus for selection a food product for addition the oregano and the wild thyme extracts is important to have considered these issues and ideal is to choose a food product with an acid pH.

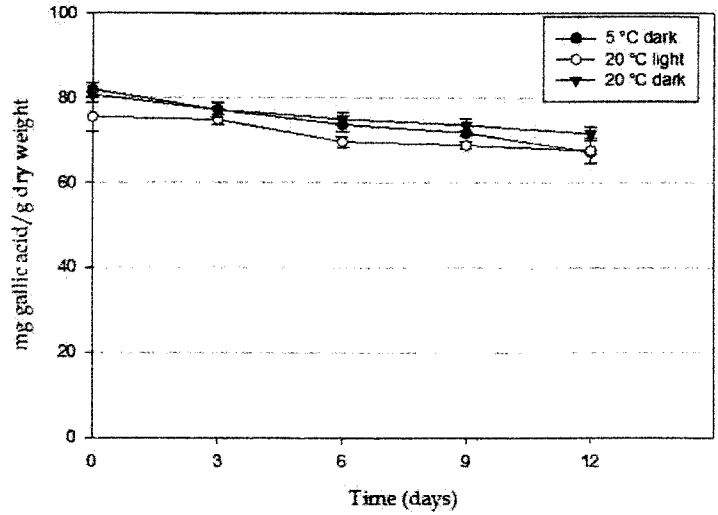


Figure 8.3 Stability of wild thyme extract at pH 5 correlated with changes of temperature and the presence of light radiation

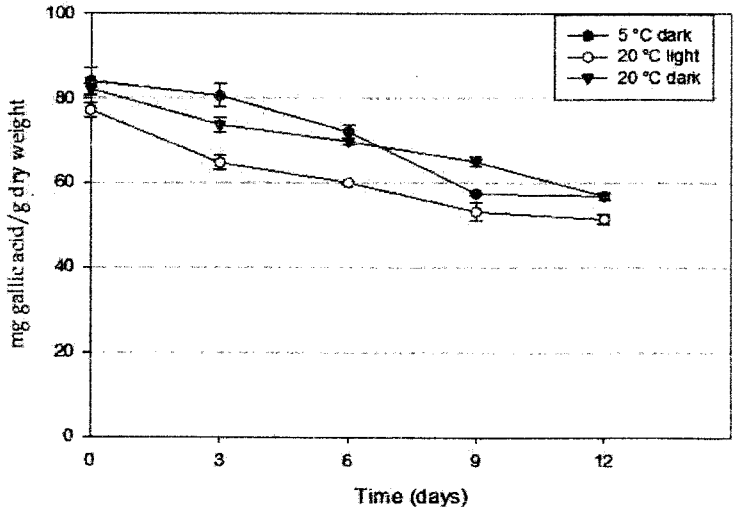


Figure 8.4 Stability of wild thyme extract at pH 7 correlated with changes of temperature and the presence of light radiation

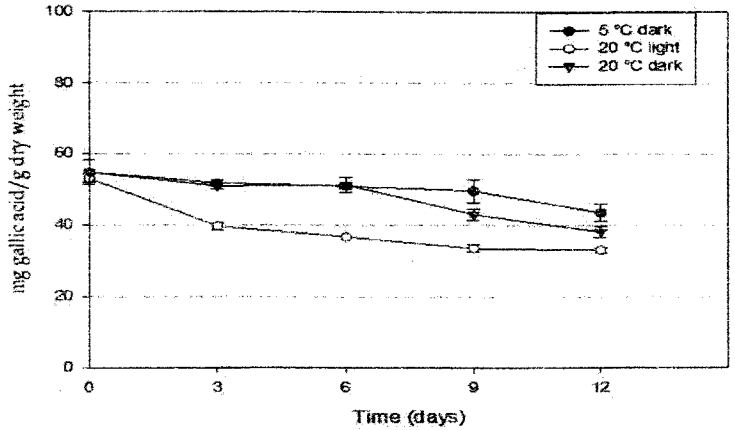


Figure 8.5 Stability of wild thyme extract at pH 9 correlated with changes of temperature and the presence of light radiation

8.3.3. Obtaining, characterization and use of microemulsions containing oregano and wild thyme extracts

Different kinds of lipids can be used to prepare emulsion-based delivery systems in both the food and pharmaceutical industries, e.g., digestible versus non-digestible oils, triglyceride oils versus essential oils, or triglyceride oils with different molecular characteristics (chain lengths, unsaturation). A number of previous studies have shown that oil type has a major impact on lipid digestion and release (McClements şi Li, 2010).

The next generation of microemulsion systems was introduced by Garti's research team (Spernath şi Aserin, 2006). These unique systems can form W/O microemulsions that can progressively transform into O/W microemulsions with no phase separation. These microemulsions with no phase separation are useful vehicles for improving solubilisation and bioavailability of bioactive compounds.

The obtaining of microemulsions with no separation phase was very important in this study. Thus, only microemulsions nut oil:Span 80:ethanol 50:30:20, respectively linoleic acid:Tween 80:ethanol 60:5:35 (figures 8.5, 8.6, 8.7, 8.8, 8.9) were obtained with no phase separation.

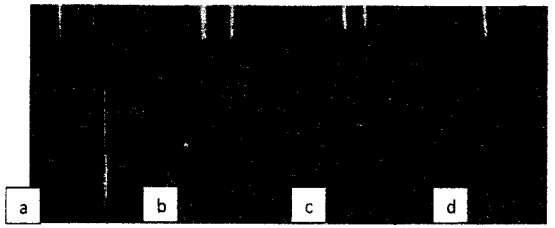


Figura 8.6. Multiphase systems - nut oil:Tween 20:ethanol
a)50:30:20; b) 50:20:30; c) 50:10:40; d) 40:10:50

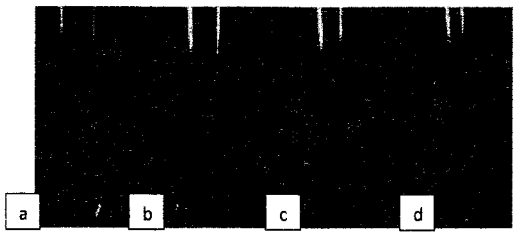


Figura 8.7. Multiphase systems - nut oil:Tween 20:glycerol
a)50:30:20; b)50:20:30; c)50:10:40; d)40:10:50

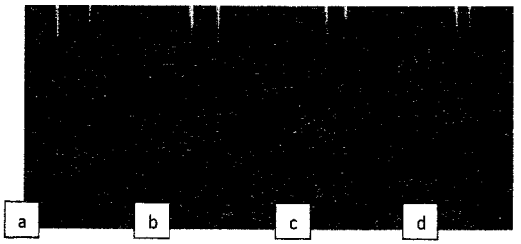


Figura 8.8. Multiphase systems - nut oil : Span 80 : ethanol a) Microemulsion - nut oil : Span 80 : ethanol 50:30:20; b) 50:20:30; c) 50:10:40; d) 40:10:50

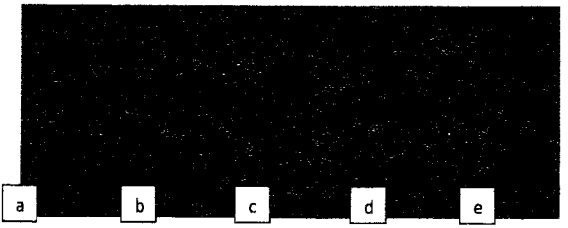


Figura 8.9. Multiphase systems - lemon oil/Tween 80/ethanol
a) 50:30:20; b) 50:20:30; c) 50:10:40; d) 40:10:50 and microemulsion linoleic acid:Tween 80:etanol e) 60:5:35

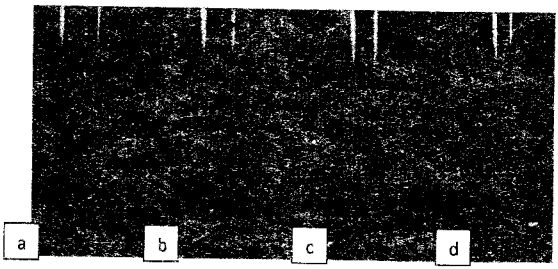


Figura 8.10. Multiphase - nut oil: Span 80: glycerol
a)50:30:20;b)50:20:30; c)50:10:40; d)40:10:50

The final objective of this study was aimed to improving the antioxidant capacity of a fruit juice supplemented with microemulsions with plant extracts (concentration in microemulsion 1mg/ml). Analysis was performed compared with a smaple of juice supplemented with synthetic antioxidants BHA and BHT (100 ppm). As blank fresh pear juice was used, obtained in laboratory with a Philips HR2744 Juicer.

The antioxidant capacity of these juice was determined by DPPH method figure 8.11.

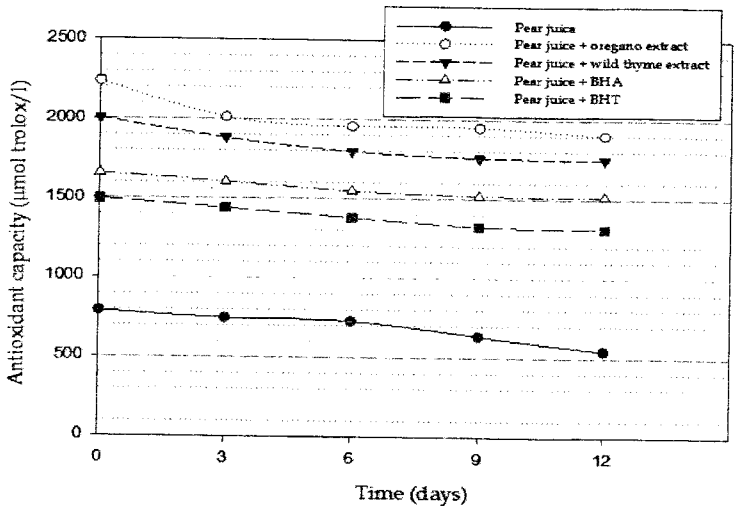


Figure 8.12 Antioxidant capacity of pear juice (control) and pear juices supplemented with antioxidants, at 5 °C for 12 days

As shown in figure 8.11 after storage at 5 °C for 12 days, oregano and wild thyme extracts improve antioxidant capacity of pear juice about 3.42 times for oregano extract and about 3.15 times for wild thyme extract. Comparing pear juice supplemented with oregano and wild thyme extracts with pear juice supplemented with BHA and BHT its observed that oregano extract improved the antioxidant capacity of pear juice about 1.25 times than pear juice supplemented with BHA and approximately 1.44 times than pear juice supplemented with BHT.

The wild thyme extract improved antioxidant capacity of pear juice approximately 1.15 times comparing with pear juice supplemented with BHA and approximatively 1.33 than pear juice supplemented with BHT.

8.4. Partial conclusions

Were studied the stability of oregano and wild thyme extract obtained by pressurized liquid extraction at different conditions of pH, temperature, presence or absence of light radiation.

Microemulsion that provides a good delivery and stabilization of oregano and wild thyme extracts is a mixture: acid linoleic/Tween 80/ethanol 60:5:35

Microemulsions containing oregano and wild thyme extracts improve de bioavailability of bioactive compounds and antioxidant capacity in food systems, at higher values compared with synthetic antioxidants BHA and BHT.

9. GENERAL CONCLUSIONS

- Oregano (Origanum vulgare L.) wild thyme (Thymus serpyllum) and tarragon (Artemisia dracunculus) are three autochtonous aromatic plants, less studied in terms of chemical composition, and are important sources of phenolic compounds with potential applications in food industry and to increase the life quality.
- Was studied the possibility of obtaining biologically active extracts from oregano, tarragon and wild thyme, using a classic extraction technique and two advanced techniques, supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE).
- The potential of the three aromatic plants studied employed three methods of determination the antioxidant capacity, namely: photochemiluminiscence method (PCL), DPPH method and TEAC method.
- Supercritical fluid extraction (SFE) using CO_2 as solvent and ethanol as modifier although leading lo lower yields, is a effective technique for extraction essential oils.
- The chemical composition of essential oils obtained by supercritical fluid extraction were characterized by gas chromatography mass spectrometry (GC-MS). Were indentified 26 bioactive compounds responsible for antioxidant capacity such as: thymol, carvacrol, germacrene D, 1.8 cineole, linalool, borneol.
- Pressurized liquid extraction (PLE) has proven the most effective technique for extraction of bioactive compounds of oregano, wild thyme and tarragon.
- It has been demonstrated that the extraction yield and extracts composition varies depending on extraction process parameters, the most important of them are solvent and extraction temperature.
- The use of an LC-MS/MS method allowed the characterization of the phenolic compounds on PLE extracts. Thirty different compounds could be tentatively assigned by using this method, some of them described for the first time in these plants. Oregano extracts were mainly characterized by the presence of

phenolic acids, mainly rosmarinic and caffeic ethyl ester acids. Extracts from tarragon were particularly rich on caffeoyl and dicaffeoylquinic acids, as well as on other flavonoids, whereas wild thyme presented the most complex chemical profile including phenolic acids and different glycosilated flavonoids and aglycons.

- Essential oils and extracts from aromatic plants, oregano, tarragon and wild thyme are not presented, at the concentrations tested, antimicrobial activity against Gram positive, Gram negative bacteria and against *Saccharomyces cerevisiae*.
- Preliminary results demonstrate that *Origanum vulgare* L. extract is a suitable stimulative and protective mixture of phenolic compounds upon probiotic bacteria, *Lactobacillus acidophilus* and *Lactobacillus casei* (Chr. Hansen commercial starters).
- The stability of oregano and wild thyme extract obtained by pressurized liquid extraction at different conditions of pH, temperature, presence or absence of light radiation were studied and also the possibility of solubilization and increasing the bioavailability in food systems by microemulsions.
- The obtained microemulsions can be use for addition in food products, and improve the antioxidant capacity at higher values compared with synthetic antioxidants BHA and BHT.

10. ORIGINAL CONTRIBUTIONS AND PERSPECTIVES

The PhD thesis researches bring the following original contributions:

- The total phenolic content and antioxidant capacity of three different plants from Romania were measured. The results showed the high phenolic content and antioxidant capacity and provide useful information like the potential use of plants as a natural source of antioxidants and as a value-added product in the preparation of functional food ingredients and/or for enrichment of certain products.

- Were used two of the most advanced extraction methods, supercritical fluid extraction (SFE) and pressurized liquid extraction to obtain essential oils and extracts rich in polyphenols and with antioxidant capacity.
- Were studied and optimized extraction conditions to identify optimal processing conditions in order to improve extraction yields and the extracts composition.
- Modern analytical techniques were applied based on chromatography coupled with mass spectrometry, for chemical composition characterization of extracts. These data have theoretical and practical importance contributing to the development of this field.
- It has been demonstrated the beneficial role of oregano extract obtained by pressurized liquid extraction, to stimulate and extend the functionality of probiotic bacteria viability *in vitro* and *in vivo* simulated conditions.
- It has been studied the possibility of using plant extracts from oregano and wild thyme as natural antioxidants, improving by microemulsions the stability, solubility and bioavailability in food systems.

Further *in vivo* investigations are still necessary to prove the functionality of aromatic plant extract and their implications for improving the life quality.

11. RESULTS DISSEMINATION

The results obtained in the frame of the doctoral stage have been disseminated in 3 scientific articles and 2 papers presented in representative international conference.

A. Articles published in ISI journals

Miron, T. L., Plaza, M., Bahrim, G., Ibáñez, E., & Herrero, M. (2011). Chemical composition of bioactive pressurized extracts of Romanian aromatic plants. *Journal of Chromatography A*, 1218(30), 4918-4927.

Aida Vasile, **Tudor Lucian Miron**, Daniela Paraschiv, Gabriela Bahrim, Stefan Dima (2011). The enhancement of the growth ability and the viability of some probiotic bacteria in media with wild *Origanum vulgare* L. extract. *Romanian Biotechnological Letters (Corrected proof)*

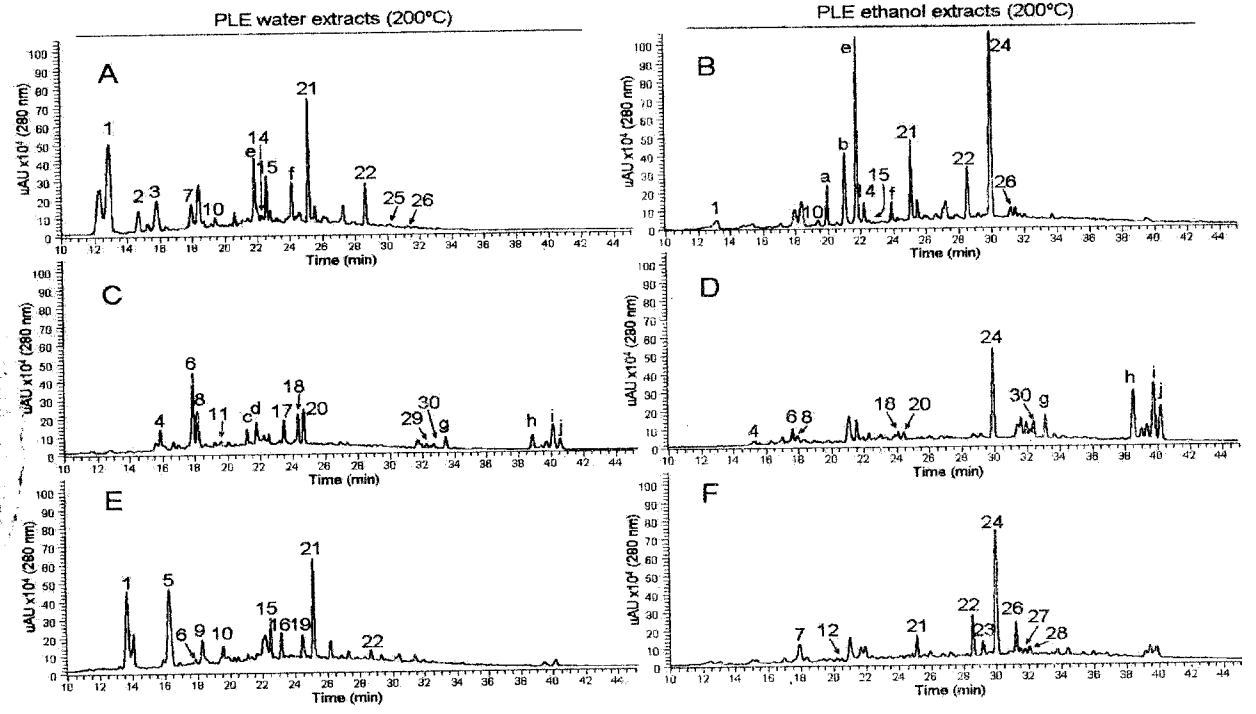
B. Article published in Indexing Data Basis journals

Miron, T.L., Gazi, I., Plaza del Moral, M. (2010). Romanian aromatic plants as sources of antioxidants. *Innovative Romanian Food Biotechnology*, (6), 18-24, http://www.bioaliment.ugal.ro/revista/6/Paper%2063.pdf

C. Scientific contributions presented in representative international conferences

T.L. Miron, G. Bahrim, E. Ibáñez, M. Herrero. (2010). Chemical and functional characterization of pressurized liquid extracts (PLE) from Romanian aromatic plants. *Cuarta Reunion de Expertos en Tecnologias de Fluidos Comprimidos*, Ciudad Real, Universidad de Castilla-La Mancha, Spain

T.L. Miron, M. Plaza, E. Ibáñez, M. Herrero (2010). Chemical composition and antioxidant activity of pressurized liquid extracts from Romanian aromatic plant, *28th International Symposium on Chromatography*, Valencia, Spain



Annex 1. LC-DAD-MS/MS chromatograms (280 nm) of the different extracts obtained using PLE at 200°C from Romanian oregano (A, B), tarragon (C, D) and wild thyme (E, F).