“BABEŞ-BOLYAI” UNIVERSITY, CLUJ-NAPOCA
FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING

PhD THESIS SUMMARY

RESEARCHES REGARDING THE INFLUENCE OF MICROWAVES ON BIOACTIVE COMPOUNDS FROM *SATUREJA HORTENSIS* L. AND *OCIMUM BASILICUM* L.

Scientific advisor
Prof. PhD Constantin Măruțoiu

PhD student
Ildikó Broş (married Lung)

Cluj-Napoca
-2012-

TABLE OF CONTENTS

Keywords: bioactive compounds, chromatography, UV-Vis, extraction, microwave.

ABBREVIATIONS

AAPH	2,2'-azobis(2-amidino-propane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AUC	area under curve
BI	irradiated basil
BR	reference basil
CI	irradiated savory
CR	reference savory
DPPH	2,2-diphenyl-1-picryl hydrazyl radical scavenging
FID	flame ionization detector
GC	gas chromatography
HPLC	high- performance liquid chromatography
IM	chamber with microwaves (of irradiation)
IR	reference chamber
M	maceration
MAE	microwave-assisted extraction
MS	mass spectrometry
NTS	diphenylboriloxyethylamine
ORAC	oxygen radical absorbance capacity assay
PDA	photodiode array detector
PEG	polyethylene glycol
TEAC	trolox equivalent antioxidant capacity assay
TLC	thin-layer chromatography
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UAE	ultrasound-assisted extraction
UV	ultraviolet
VIS	visible

INTRODUCTION

Progress in the field of molecular biology and of genetic forms of handling offers promising perspectives for intensification of biosynthesis of secondary compounds acting at regulatory genes. Even though there are often relevant differences in the synthesis and accumulation of secondary metabolites in different tissues or in different stages of plant development, the genome of each cell contains the information required to trigger the full potential of secondary metabolism characteristic of species.

Recent studies [1-3] indicate that the presence of microwave radiation affects living systems, even at power levels below the normatives.

PERSONAL CONTRIBUTIONS

The thesis entitled "Researches regarding the influence of microwave field on the bioactive compounds from *Satureja hortensis* L. and *Ocimum basilicum* L." has a main goal to determine the influence of the microwave field on the bioactive compounds present in the mentioned plant species.

1. A major element of innovation of this thesis is the growth of the studied plants, in specially designed microwave anechoic chambers (a reference chamber and a chamber with microwave field) and the tracking of the bioactive compounds present in these plants, both irradiated and non-irradiated samples.
2. Another study was performed in order to find the optimum extraction method for the volatile oils and the polyphenolic compounds (flavonoids and polyphenolcarboxylic acids). The method was then employed for the extraction of the mentioned compounds from the plants which were grown in the microwave field. An optimum extraction method was necessary because of the very small quantities of bioactive compounds obtained from the plant samples, as the duration of the experiments is only five weeks after plant germination. In the study was introduced a new microwave extraction method in pulses using a prototype device, designed and built in INCDTIM, Cluj-Napoca.
3. For the analysis of the obtained compounds, classical analysis methods were used, both on the irradiated and non-irradiated (reference) plant samples.

4. The element of innovation with a high practical importance is the determination of the influence of the microwave field on the following classes of bioactive compounds: assimilatory pigments, volatile oils and polyphenolic compounds. At current time, the scientific literature does not present a concrete conclusion over the influence of the microwave field on the mentioned compounds.
5. Another element of innovation is the modification of the antioxidant property of the irradiated plants compared with the non-irradiated ones.
6. As a conclusion of these studies, it can be stated that, in the microwave irradiated plant samples, the volatile oil quantities and polyphenolic compounds are higher compared with the quantities obtained from non-irradiated samples. Moreover, the assimilative pigment quantities are lower in the microwave irradiated plants.

Due to its objectives, performed studies and conclusions, this thesis meets the modern global trends in the field.

EXPERIMENTAL PART

CHAPTER 4

SELECTION THE EFFICIENT EXTRACTION METHOD OF BIOACTIVE COMPOUNDS FROM SATUREJA HORTENSIS L. END OCIMUM BASILICUM L.

4.1. Determination of efficient method for extraction of volatile oils from studied plants

Basil (Ocimum basilicum L.) and garden savory (Satureja hortensis L.), two aromatic herbs with high contents of essential oils, are widely used in gastronomy because of their distinctive strong flavor.

4.1.1. Extraction of volatile oils

The following solvents and solvent mixtures were used for extraction of essential oils: E1 - hexane, E2 - ethyl ether, E3 - ethanol, E4 - hexane - ethyl ether (1:1, v / v), E5 - ethyl ether - ethanol (1:1, v/v), E6 - hexane - ethyl ether - ethanol (1:1:1, v/v).

Extraction techniques empoyed were: maceration, ultrasound extraction and extraction in microwave power field.

4.1.2. Qualitative analysis of essential oils

The quality of the extracts was determined by chromatographic methods (TLC, GC-FID).

4.1.2.1. Analysis by thin-layer chromatography

Basil and savory extracts obtained with the system E6 were subjected to analysis: hexane - ethyl ether - ethanol (1:1:1, v/v) obtained by three techniques: maceration, ultrasound and microwave. TLC analysis were performed on Sil G F_{254} HPTLC plates. Before using, the plates were conditioned with methanol and dried at 110°C, for 3 hours. The samples were applied with an Linomat 5 semi-automatic applicator, as 5 mm bands. 20 µL volumes were applied for plant extracts and 7 µL for standards. The toluene - ethyl acetate (93:7, v/v) was used as mobile phase. Visualization was achieved by spraying plates with anisaldehyde and heating for 3 min at 110°C, when the bands turn red - blue. The plates obtained in this way were analyzed in visible light and at 365 nm with a UV lamp [182].

From the chromatograms obtained for savory, respectively basil, by all three extraction methods, we did not observe any difference from the quality point of view, which indicates that the volatile oils are not degraded by any of the used methods.

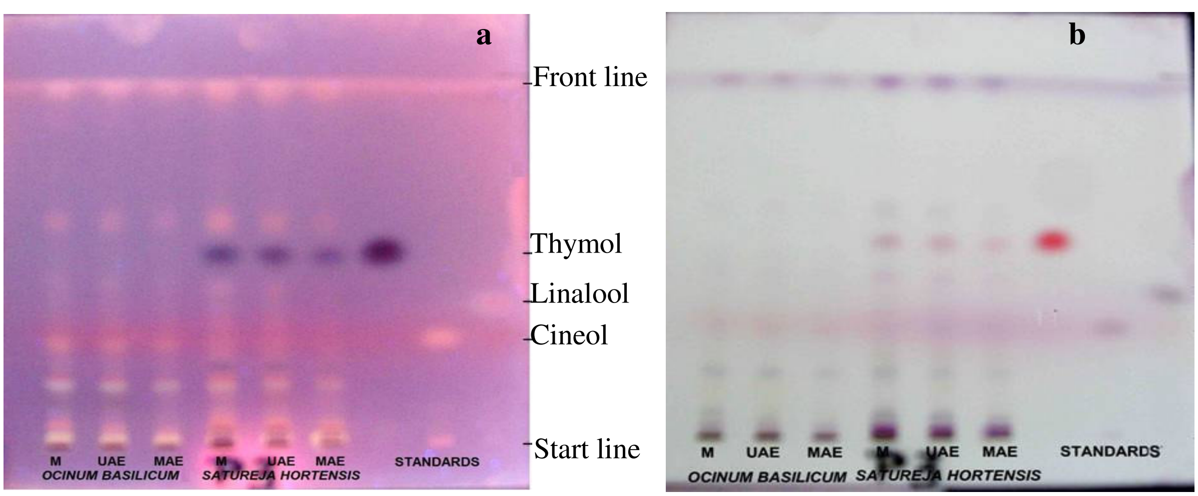


Figure 15. Cineole, linalool and thymol identification in the extracts from Satureja hortensis L. and Ocimum basilicum L. Stationary phase: HPTLC Sil G F254. Mobile phase: toluene – ethyl acetate (93:7, v/v). Detection: with anisaldehide at 365 nm (a) and in visible light (b).

4.1.2.2. Analysis by gas chromatography coupled with flame ionization detector

GC-FID analysis was performed using a Shimadzu GC-2010 gas chromatograph with flame ionization. The compounds were separated on a methyl silicone OV-17 (2 m) column. The oven temperature was scheduled as follows: 2 min at 80°C, 4°C/min for heating in the range 80°C to 200°C, 1 min at 200°C and then 20°C/min to 260°C, where was maintained 35 min. The detector and the injector temperatures were 240°C, 260°C respectively. Helium was used as carrier gas with a flow rate of 15 mL/min and injected volume was 2 µL [182, 184, 185].

Based on the results obtained by GC-FID we chose the most efficient extraction conditions for the essential oils from savory and basil: ultrasonic extraction in hexane - ethyl ether (1:1, v / v) for savory and extraction in microwave field in diethyl ether - ethanol (1:1, v / v) for basil.

4.2. Determination of efficient method for extraction of polyphenolic compounds from studied plants

The obtained Polyphenolic extract can be analyzed globally - non-specifically by determining total polyphenols as well as specific ones.

4.2.1. Extraction of polyphenolic compounds

For each sample we extracted 0.5 g powder plant using 40 mL hydroalcoholic extraction mixture of different concentrations (Table 2). Before refluxing, ultrasound and extraction in microwave field, the vegetable powder was left for 30 minutes to macerate.

Table 2. The composition of extraction systems used: mixture of ethanol - water (v/v)

ES1	ES2	ES3	ES4	ES5	ES6	ES7
100:0	90:10	80:20	70:30	60:40	50:50	40:60

Extraction techniques used:

a) Maceration was done in 14 days at 30°C.

b) Refluxing occurred at temperatures higher than 70°C, for 30 minutes.

c) Ultrasound-assisted extraction, using an Elmasonic S ultrasonic bath was carried out at a temperature of 70°C, for 30 minutes.

Microwave-assisted extraction was carried out in a dynamic substance processing installation based on power microwave fields (INCDTIM) [181]. Extraction was performed at a power of 900 W, at a fixed temperature of 70°C, in atmospheric pressure with a fill factor of 40% for 2 minutes. The total extraction time was longer than the action of microwaves because the system needed additional time of cooling.

4.2.2. Qualitative analysis of polyphenolic compounds

4.2.2.1. Analysis by thin-layer chromatography

Identification of polyphenolic compounds was performed for Satureja hortensis L. extracts obtained by maceration, refluxing and extraction in microwave power field [192].

The prepared extracts (ES3, ES7) were applied using a Hamilton microsyringe 10 µL on chromatographic plate (10 X 10 cm HPTLC) with silica gel. Both extracts and standards were applied as a band of 0.7 cm to 1.5 cm from the bottom and lateral of the plate. The system: n-hexane - ethyl acetate - formic acid (30:20:1,5 v / v) was used as mobile phase. Methanolic solution of luteolin (1 mg/mL) and rosmarinic acid (0.7 mg/mL) were used for identification. The plates were developed over a distance of 8 cm in chromatographic rooms previously saturated for 15 minutes. Visualization of the plates was carried out by spraying the plates with NTS / PEG 400 (diphenylboriloxyethylamine/polyethylene glycol 400) at 365 nm.

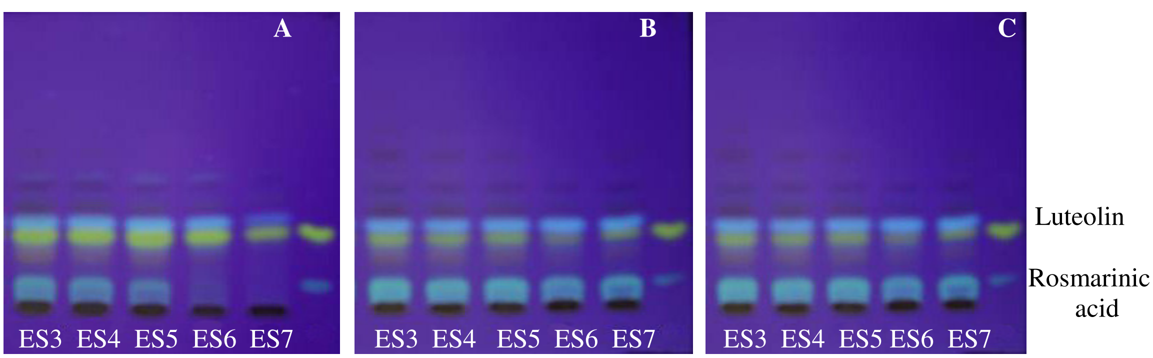


Figure 23. Chromatogram of *Satureja hortensis* L. extracts (ES3-ES7): A-maceration, B-refluxing, C-extraction in microwave field, along with standard solutions. Stationary phase: TLC Sil G. Mobile phase: n-hexan - acetat de etil - acid formic (30:20:1,5 v/v). Detection: with NTS/PEG 400 at 365 nm.

4.2.2.2. Analysis by high-performance liquid chromatography

For qualitative analysis of polyphenolic compounds we used savory and basil extracts obtained by refluxing with the ethanol - water (70:30, v/v) system. The chromatographic analysis was carried out with a Shimadzu system consisting of a binary pump, degasser, autosampler, thermostat set at 30°C, PDA and MS detectors. Separation was carried out on a RP-18 column with a mobile phase of acetonitrile and water (1% formic acid). Elution begins with a linear gradient starting with acetonitrile from 5% to 42% acetonitrile in 5 minutes, followed by elution with a linear gradient of acetonitrile up to 35% in 25 minutes. Mobile phase flow rate was 0.43 mL/min and injection volume 10 μL.

From the qualitative analysis of Ocimum basilicum L. extracts, by comparing the retention times, the wavelengths of the corresponding maxims of standards with those of the compounds in the extracts and their molecular weight, the following compounds were identified: caffeic acid, rosmarinic acid, luteolin, apigenin and naringenin.

From the qualitative analysis of Ocimum basilicum L. extracts, by comparing the retention times, the wavelengths of the corresponding maxims of standards with those of the compounds in the extracts and their molecular weights, the following compounds were identified: caffeic acid, rosmarinic acid.

4.2.3. Quantitative analysis of polyphenolic compounds

Quantitative analysis of polyphenolic compounds was performed by two methods of analysis: spectrophotometric and chromatographic.

4.2.3.1. Spectrophotometric analysis

4.2.3.1.1. Spectrophotometric analysis of total flavonoids

Total flavonoid content was determined spectrophotometrically after reaction with $AlCl_3$ reagent [193].

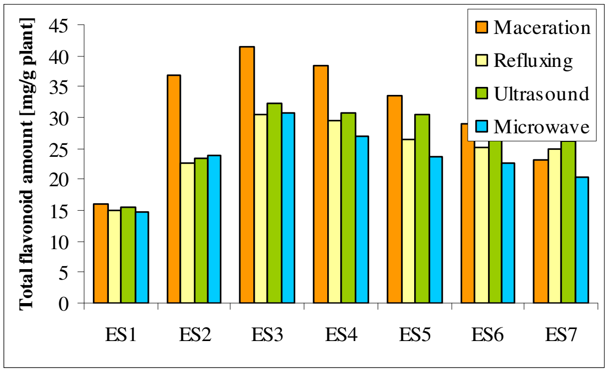


Figure 30. Comparison chart of the total amount of flavonoids depending on the solvent and extraction technique used for *Satureja hortensis* L. extracts.

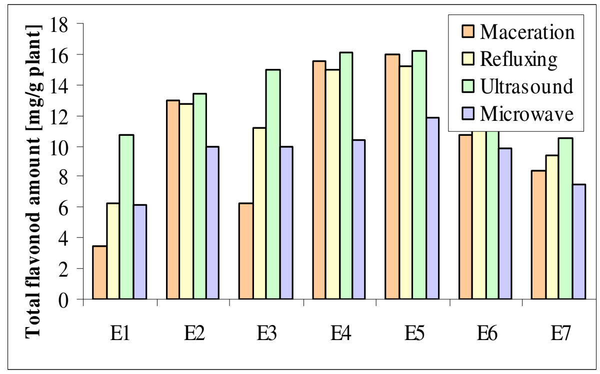


Figure 31. Comparison chart of the total amount of flavonoids depending on the solvent and extraction technique used for *Satureja hortensis* L. extracts.

For extraction of flavonoids from Satureja horetnsis L., extraction by ultrasound using EtOH-H_2O solvent mixture (80:20, v/v), while for the extraction of flavonoids from Ocimum basilicum L., extraction by ultrasound using EtOH-H_2O solvent mixture (60:40, v/v), have proven to be the best method in terms of the quantity of the extracted compounds and the required extraction time .

4.2.3.1.2. Spectrophotometric analysis of polyphenolcarboxylic acids

Total polyphenolcarboxylic acids content (caffeic acid type) was determined spectrophotometrically after reaction with Arnow's reagent [194].

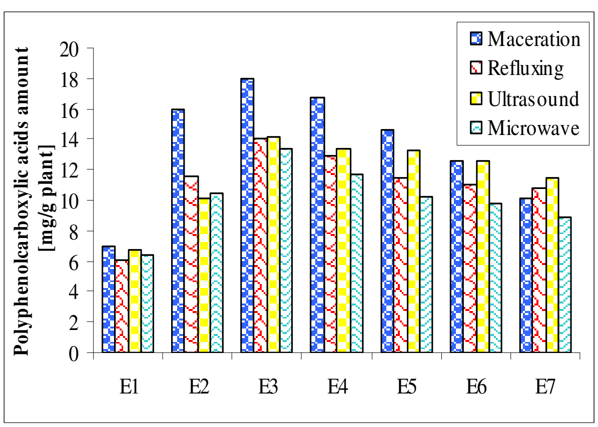


Figure 33. The amount of polyphenolcarboxylic acids extracted from *Satureja hortensis* L. by different techniques using various extraction systems.

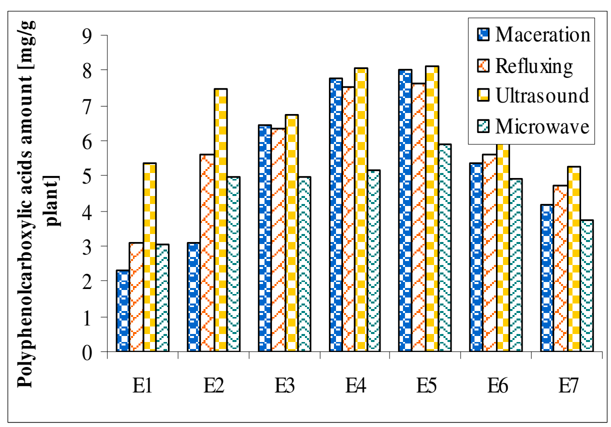


Figure 34. The amount of polyphenolcarboxylic acids extracted from *Ocimum basilicum* L. by different techniques using various extraction systems.

The amount of polyphenolcarboxylic acids extracted from *Satureja hortensis* L. and *Ocimum basilicum* L. differs by extraction technique and extraction system used. Thus, polyphenolcarboxylic acids from savory is better extracted with the ES3 system, while for basil ES5 extraction system is the most efficient. As best extraction technique is maceration, followed by ultrasound extraction, refluxing and microwave extraction.

4.2.3.2. Analysis by thin-layer chromatography

From the equation of of rosmarinic acid and luteolin calibration lines we calculated their concentrations in the extracts of Satureja hortensis L. and the results are presented in the charts shown in Figures 37 and 38.

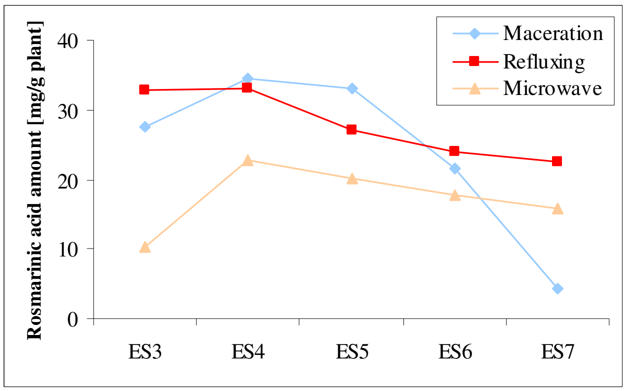


Figure 37. Comparison chart of rosmarinic acid content in the studied extracts.

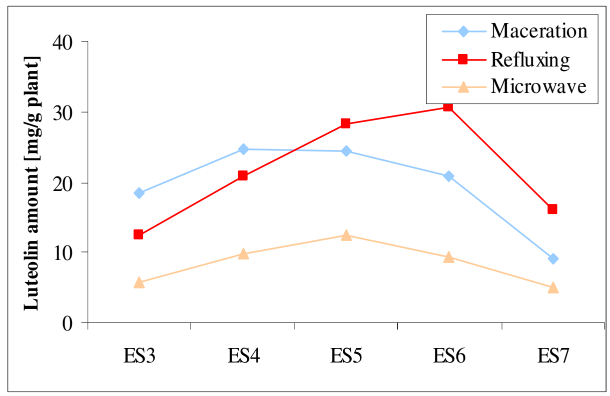


Figure 38. Comparison chart of luteolin content in the studied extracts..

Comparing the extraction techniques, we can see that the highest amount of luteolin and rosmarinic acid is extracted by refluxing, followed by maceration.

4.2.3.3. Analysis by high-performance liquid chromatography

Results obtained from the quantitative analysis are represented in the graphs below.

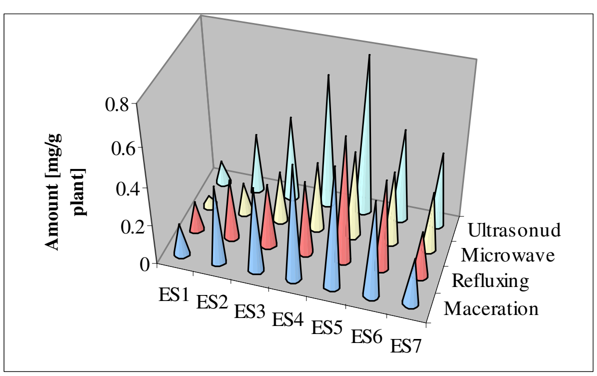


Figure 44. Caffeic acid content extracted from *Satureja hortensis* L.

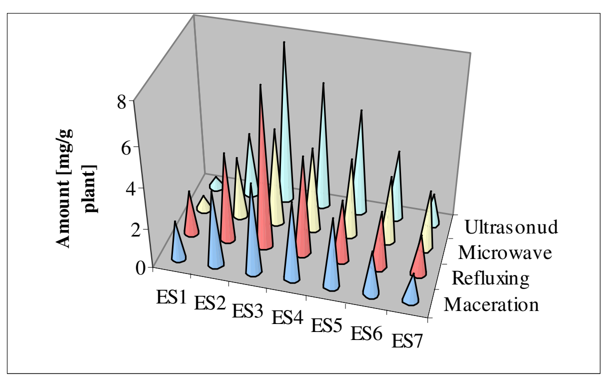


Figure 45. Rosmarinic acid contend extracted from *Satureja hortensis* L.

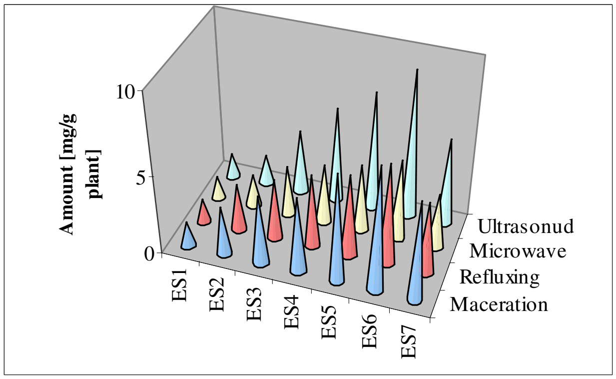


Figure 46. Luteolin content extracted from *Satureja hortensis* L.

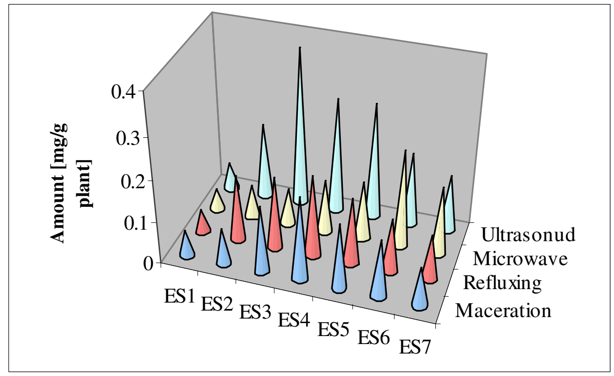


Figure 47. Naringenin content extracted from *Satureja hortensis* L.

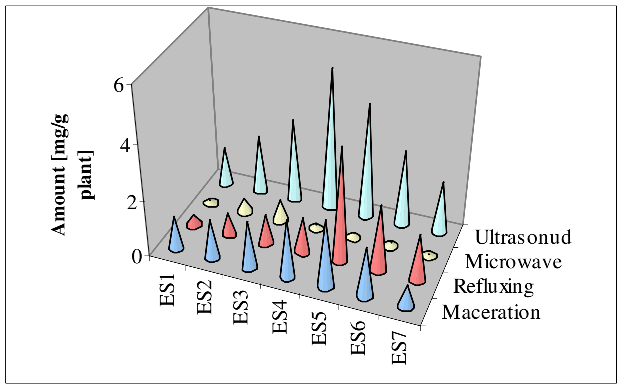


Figure 48. Apigenin content extracted from *Satureja hortensis* L.

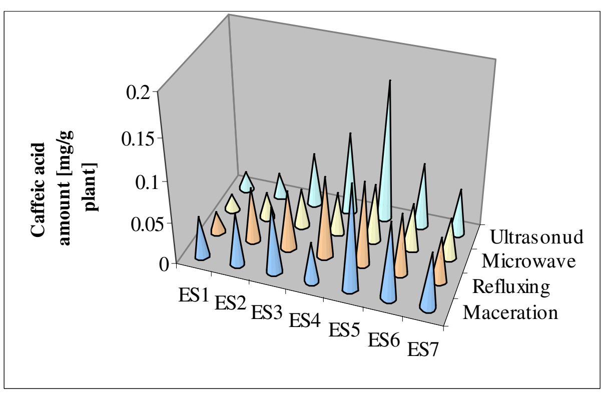


Figure 49. Caffeic acid content extracted from *Ocimum basilicum* L.

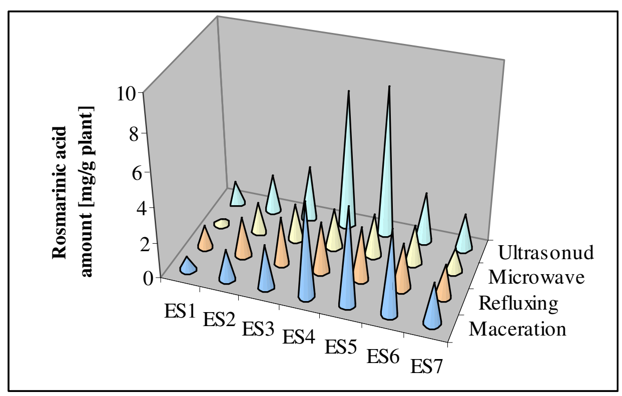


Figure 50. Rosmarinic acids content extracted from *Ocimum basilicum* L.

The composition of these extracts, does not vary from quality point of view with the used extraction techniques. The extracted quantity depends on extraction solvent used and the type of polyphenolic extracted compound as well as the used extraction technique .

CHAPTER 5
THE INFLUENCE OF MICROWAVE FIELD ON BIOACTIVE COMPOUNDS FROM *SATUREJA HORTENSIS* L. AND *OCIMUM BASILICUM* L.

5.1. Studied plant growth in microwave field

The plants taken into study were grown in the laboratory, seeds from the ARO company. At three weeks after seeding, vessels with sprung plants were placed in two identical anechoic chambers [205], one being the reference chamber and the other with the active microwave field. (Figure 51). This lot was numbered 1. Another lot of plants (only basil) was introduced in the chambers for irradiation at 20 weeks after seeding (another stage of development) - lot 2.

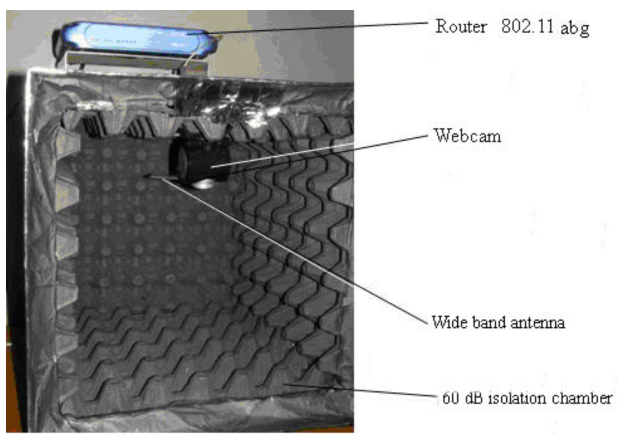


Figure 51. Installation to stimulate the plant development by applying a low power electromagnetic field.

In the first chamber (IR) we placed the reference samples and in the second (IM) chamber we place the irradiated samples, with microwaves at a frequency of 2.45 GHz and the power of 14 dBm, radiation coming from a wireless router via a specially designed antenna. Irradiation was performed over a period of two weeks, each time, after which the plants were removed from the chamber and raised in the room.

5.2. Determination of assimilating pigments

Determination of chlorophylls and carotenoids from the reference and subject to irradiation plants was performed only on basil, because savory was more sensitive to the presence of the microwave field and many plants have died either in irradiated chambers either after having been removed from them.

Plant samples (1 g) were homogenized with an Ultraturrax in the presence of 20 mL 90% acetone in water and then left for two hours with stirring. Repeat this extraction with 10 mL 90% acetone in water to plant material bleaching. Samples was filtered and Vis spectrum is registered with a UV-Vis spectrophotometer Jasco V-530 type.

5.2.1. Determination of chlorophyll pigments

The calculation of chlorophyll pigments concentration is carried out by reading the absorbances at 663, 648 and 470 nm wavelengths, according to the following formula:

chlorophyll a (mg/g wet weight) = (11,75 x A 663 – 2,35 x A 645) x 50/ 500

chlorophyll b (mg/g wet weight) = (18,61 x A 645 – 3,96 x A 663) x 50 / 500

Chlorophyll a and b content in the analyzed samples is presented in the chart below.

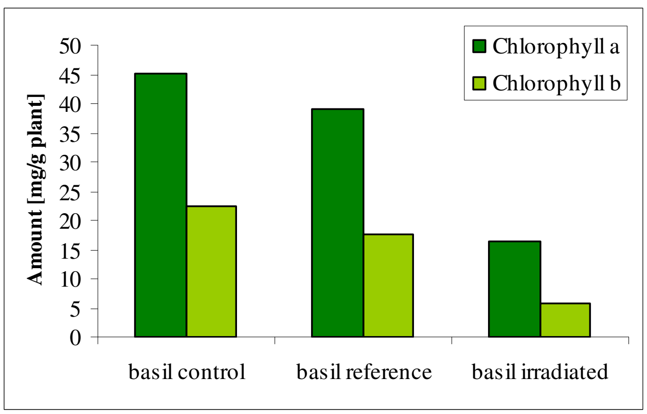


Figure 58. Chlorophyll a and b content from analyzed plants.

The content of chlorophylls a and b is higher for control plant taken from nature than the rest of the samples analyzed and chlorophyll content is always higher than the chlorophyll b. Also, chlorophylls a and b content from plants subjected to microwaves was lower than in non-irradiated plants.

5.2.2. Determination of carotenoidic pigments

Carotenoid pigments were analyzed by high performance liquid chromatography (HPLC). HPLC system consisted of LC20 AT Shimadzu pumps and Shimadzu PDA detector. YMC C30 column (25 cm x 4.6 mm, 5 mm particle diameter) was used. Mobile phase consisted of two solvent mixtures: A - methanol / methyl tert-butyl ether / water (81/15/4) and B - consists of the same components in relation to 6/90/4. A linear gradient from 1% B to 100% B in 90 min was used. Identification of carotenoids was based on comparison with retention times of standards and absorption spectra.

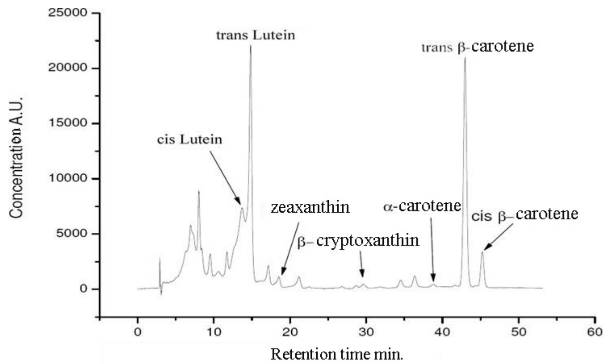


Figure 60. HPLC chromatogram for carotenoids from acetone non-irradiated basil extract.

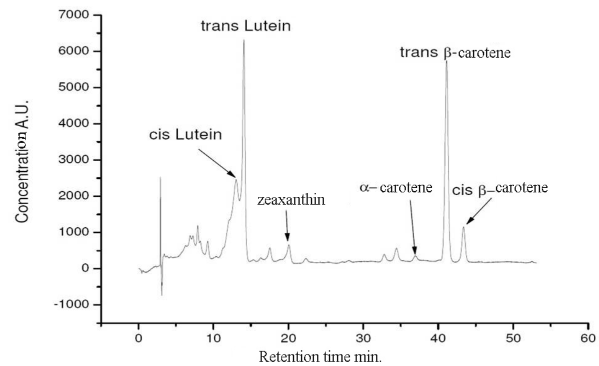


Figure 61. HPLC chromatogram for carotenoids from acetone irradiated basil extract.

It can be observed that the major carotenoid pigments in the samples studied are β-carotene and lutein, while zeaxanthin and β-cryptoxanthin are minority pigments.

The extract obtained was passed in ethyl ether and total carotenoid content was determined spectrophotometrically after the following relationship [210]:

$$X \text{ (mg carotenoids)} = (A \times V \times 1000)/ (2500 \times l \times 100)$$

where: A = absorption of the sample read at λ_{max} = 450 nm, V = sample volume (mL): 2500 = molar absorption coefficient (E1%) for carotenoids; l = 1 cm - length of spectrophotometer cuvette (optical path).

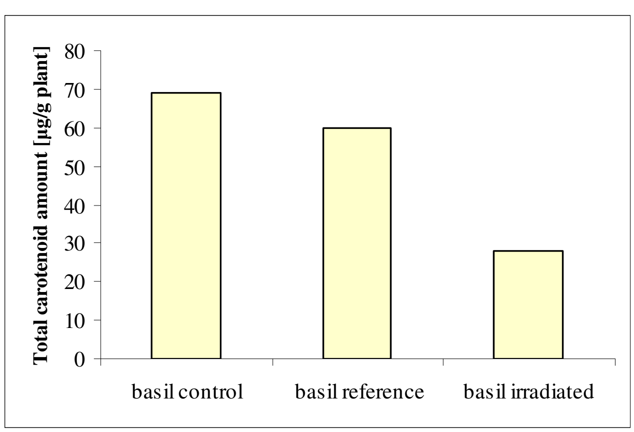


Figure 64. Total carotenoid content from studied plants.

It can be observed from the chart above that the values obtained for total carotenoids in control plant extract taken from nature is greater than the other samples analyzed. Also, the total amount of carotenoids from plants subjected to microwaves was smaller than in non-irradiated plants.

5.3. Determination of the effect of microwave field on volatile oils from irradiated plants

The plant material obtained was processed according to the protocol set out in Chapter 4, using efficient methods for the extraction volatile oils.

5.3.1. Qualitative analysis of volatile oils

The analysis of volatile oils from irradiated plants subjected to with microwave irradiation and non-irradiated savory was performed by thin layer chromatography and gas chromatography coupled with mass spectrometry.

5.3.1.1. Analysis by thin-layer chromatography

Chromatographic analysis was performed according to section 4.1.2.1., using the Sil G F_{254} HPTLC plates, the mixture of toluene - ethyl acetate (93:7, v/v) as mobile phase, spraying with anisaldehyde and inspection in Vis and UV at 365 nm.

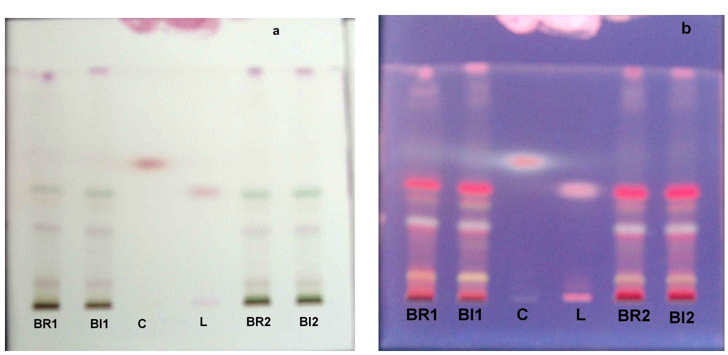


Figure 65. Chromatograms of BR1, BR2 and BI1, BI2 extracts (C – cineol, L – linalool). Stationary phase: HPTLC Sil G F254. Mobile phase: toluene – acetate de ethyl (93:7, v/v). Detection: with anisaldehyde in visible light (a) and ultraviolet at 365 nm (b).

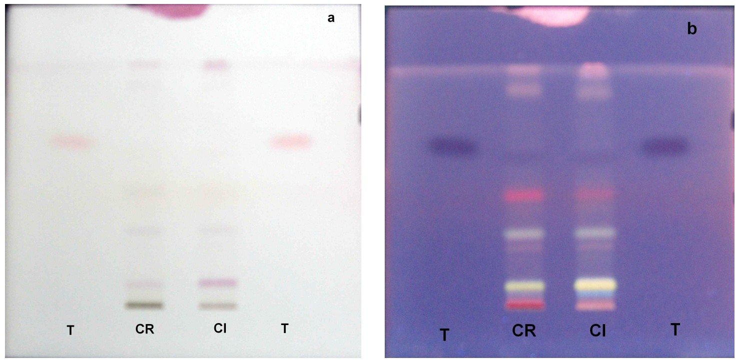


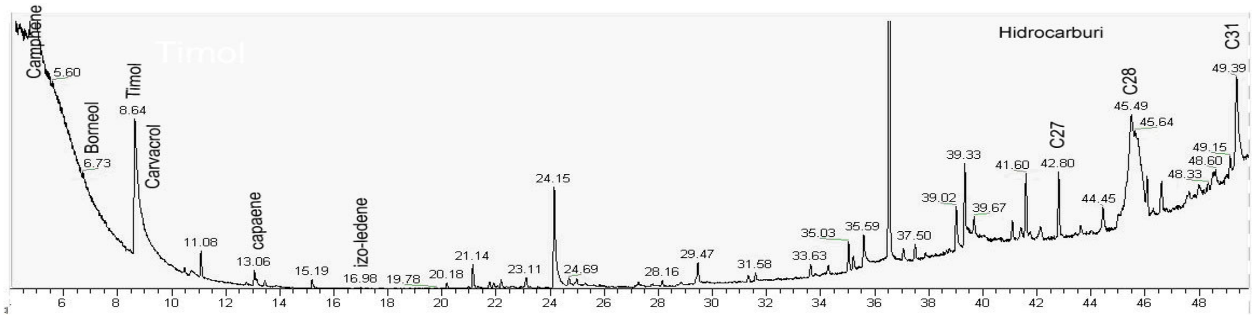
Figure 66. Chromatograms of CR and CI extracts (T – thymol). Stationary phase: HPTLC Sil G F254. Mobile phase: toluene – acetate de ethyl (93:7, v/v). Detection: with anisaldehyde in visible light (a) and ultraviolet at 365 nm (b).

At a more careful study of the chromatographic fingerprint it is observed that:

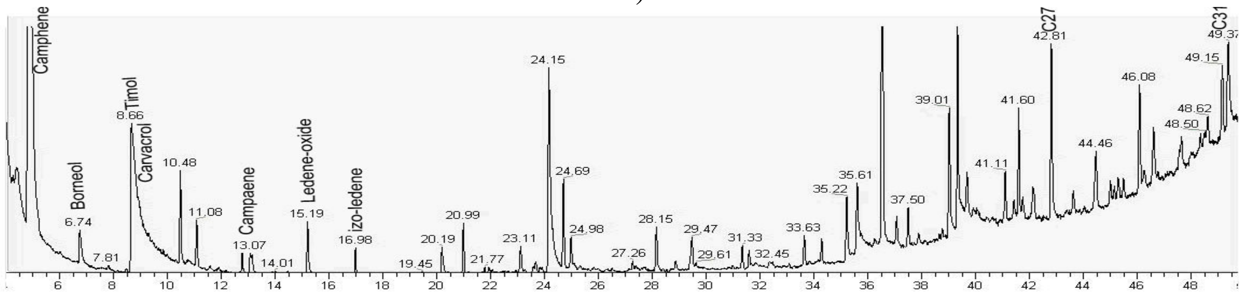
- there are no changes in chromatographic fingerprint, being present the same bioactive compounds; irradiation with microwave did not lead to new compounds;
- we observed changes in the concentration of bioactive compounds in irradiated plants compared to non-irradiated.

5.3.1.2. Analysis by gas chromatography couplet with mass spectrometry

GC-MS analysis of the obtained extract was performed using GC-MS , Thermo Electron Corporation, Ultra Trace / Polaris Q coupled system, starting from a temperature of 60°C, heating in two steps: first with 10°C / min to 120°C, and then with 4°C to 300°C. Injection temperature was 250°C and the injection volume 2 μL. Capillary column used was DB5, 30 m.



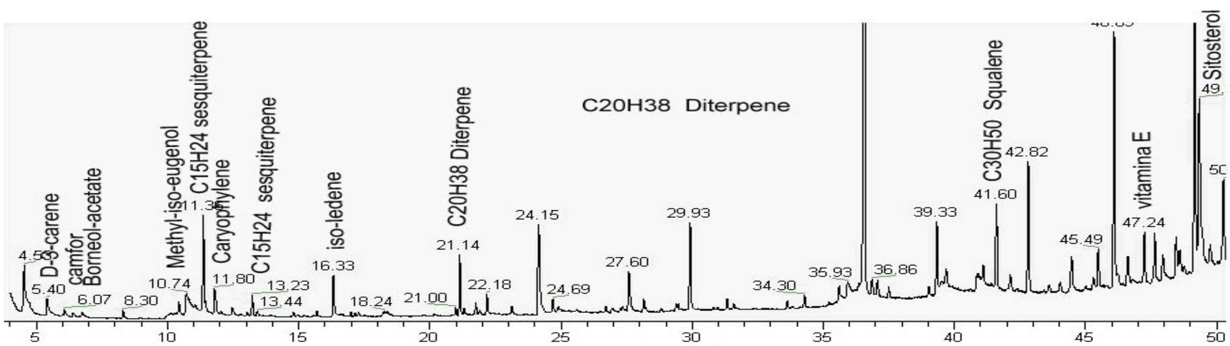
a)



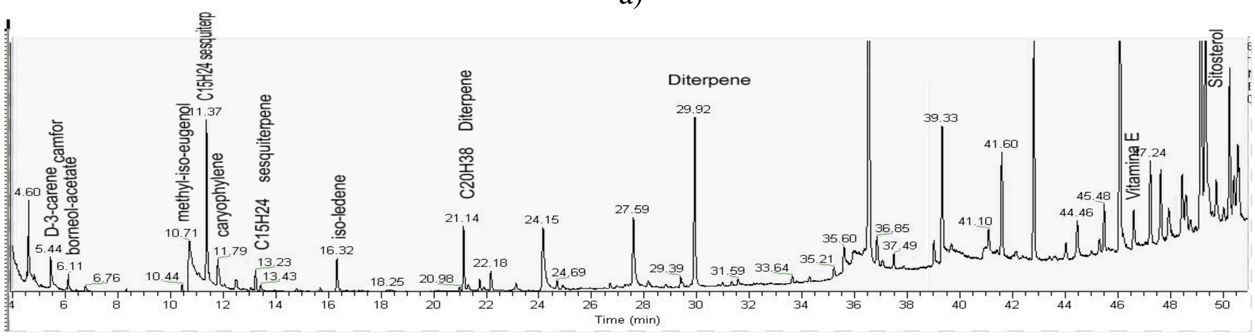
b)

Figure 67. GC-MS chromatograms of savory extracts: a) irradiated; b) non-irradiated with microwave.

From chromatograms it is observed that savory extracts contain a large quantity of thymol and carvacrol. Irradiated and non-irradiated savory containing the same major compounds, but in higher quantities in the irradiated (more camphene, borneol, thymol, carvacrol, leden oxide etc).



a)



b)

Figure 68. GC-MS chromatograms of basil extracts: a) non-irradiated; b) irradiated with microwave.

In case of irradiated basil was found a high content of terpenes, sesquiterpenes and diterpenes, in addition to hydrocarbons and other compounds. Just as with savory, irradiated plant is more rich in bioactive compounds;, results observed by other analysis techniques used.

5.3.2. Quantitative analysis of volatile oils

Quantitative determination of volatile oils was carried out by two methods, one based on calibration curves and determination of concentrations from equations of lines and the second based on determination of the percentage from each the volatile oil found in composition.

5.3.2.1. Analysis by gas chromatography coupled with flame ionization detector

Using calibration curves recorded it was determined the volatile oil composition in µg/g plant for a few volatile oils.

Table 13. The amount of volatil oils determined per gram of plant.

Plant	**Limonene** (µg/g plant)	**Terpineol** (µg/g plant)	**Citronellol** (µg/g plant)	**Thymol** (µg/g plant)	**Carvacrol** (µg/g plant)	**Eugenol** (µg/g plant)
Irradiated basil, lot 1	4,33	1,13	6,12	-	-	156,76
Non-irradiated basil, lot 1	2,87	1,10	1,15	-	-	81,00
Irradiated basil, lot 2	8,24	0,82	0,73	-	-	93,96
Non-irradiated basil, lot 2	2,22	0,82	0,62	-	-	61,44
Irradiated savory	-	-	-	1218,84	7,05	-
Non-irradiated savory	-	-	-	825,61	148,2	-

5.3.2.2. Analysis by gas chromatography couplet with mass spectrometry

The chromatograms of extracts were registered by GC-MS (Figures 71 and 72) and we determined the percentage of volatile oils content in extracts.

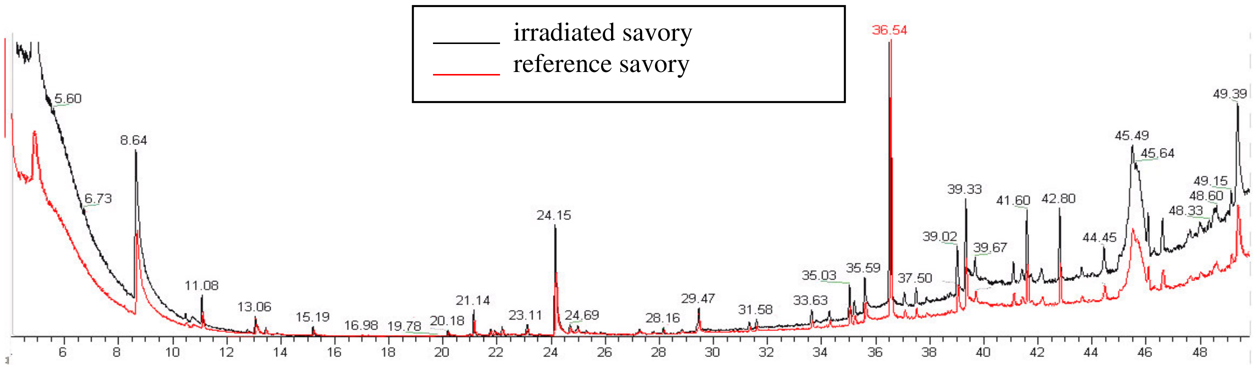


Figure 74. Chromatogram of irradiated and non-irradiated savory with microwave.

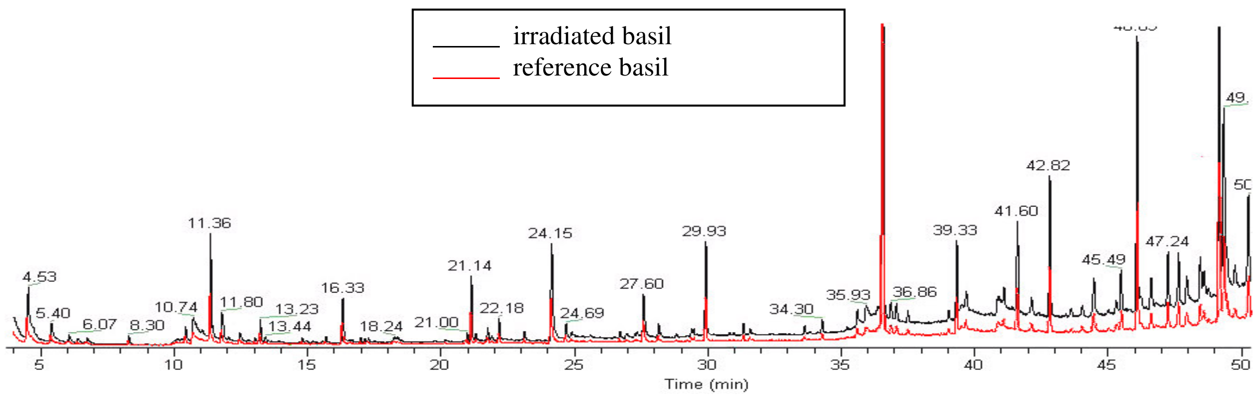


Figure 75. Chromatogram of irradiated and non-irradiated basil with microwave, lot 1.

On the recorded chromatograms we performed integration of all peaks, sum of the areas representing 100%. Starting from the peak surfaces obtained by integration, we established the procents for each of the identified compounds.

From the chromatograms recorded both by GC-FID as well by GC-MS it was found that: chemical composition of extracts from the plants irradiated and non-irradiated with microwave is the same, but the amount of volatile oils differ. It also differs depending on the stage of plant development.

In case of basil:

- the major components are: methyl iso-eugenol, eugenol, limonene, linalool, methyl p-metoxicinnamat, iso-leden;
- irradiated basil contains a much higher amount of methyl iso-eugenol (6.7 times higher) and eugenol (almost double) than the non-irradiated basil
- basil in group 2 contains borneol, which is in traces in group 1.

In case of savory:

- major component is thymol, accompanied by a very small amount of carvacrol;

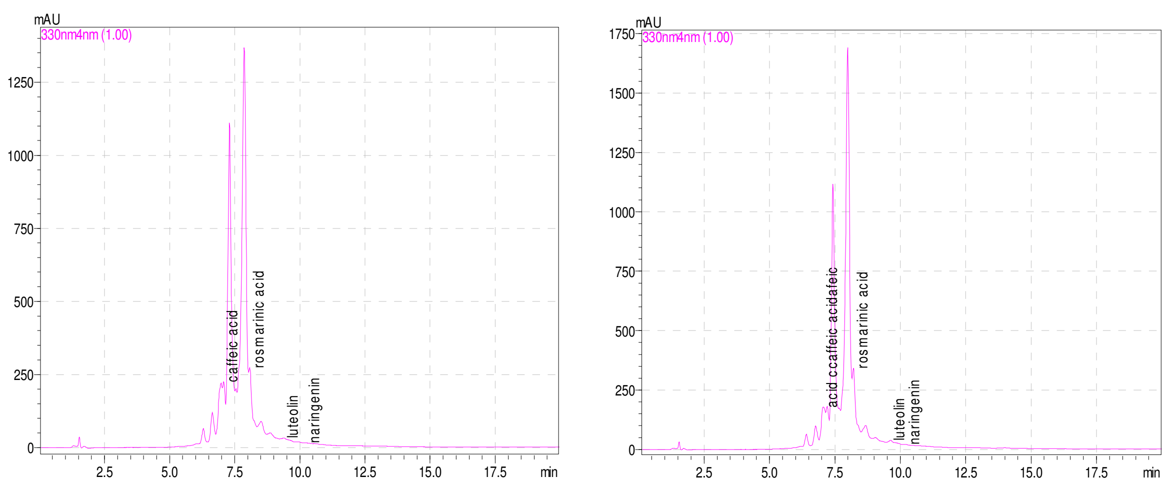
- both types of extracts containing approximately equal amounts of cariophyllene;
- in irradiated savory greatly increase the amount of tricyclens (6 times).

5.4. Determination of the effect of microwave field on polyphenolic compounds from irradiated plants

For the characterization of irradiated and non-irradiated plants with microwaves polyphenolic compounds were extracted with the extraction method previously established. For this purpose 0.5 g irradiated and non-irradiated plant were extracted in 40 mL ethanol - water (80:20, v/v) in the case of Satureja hortensis L., respectively in 40 mL ethanol - water (60: 40, v / v) in the case of Ocimum basilicum L. by ultrasound for 30 minutes. The results obtained from the plants subjected to microwave treatments were analyzed and compared with the data registered for the reference plants grown under similar conditions without being subject to irradiation.

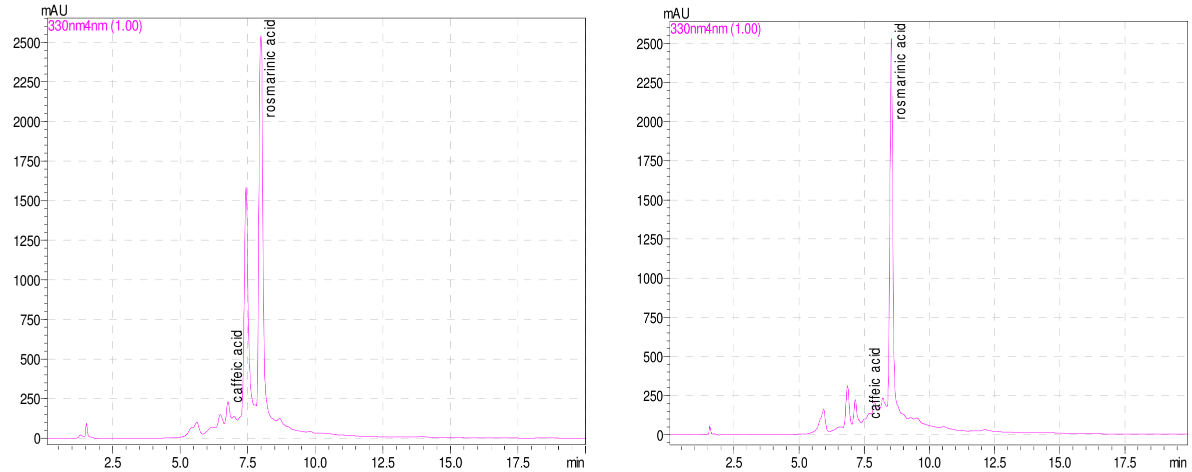
<u>5.4.1. Qualitative analysis of polyphenolic compounds</u>

Chromatograms obtained for irradiated and non-irradiated plant extracts are shown in Figures 76 and 77.



a) b)

Figure 76. Chromatograms of *Satureja hortensis* L. extracts: : a) non-irradiated, b) irradiated obtained by ultrasound with a mixture EtOH – H_2O (80:20, v/v).



a) b)

Figure 77. Chromatograms of *Ocimum basilicum* L. extracts: a) non-irradiated, b) irradiated obtained by ultrasound with a mixture EtOH – H_2O (60:40, v/v).

From the qualitative analysis we identified caffeic acid and rosmarinic acid both in the savory as well in the basil extracts. In addition to these compounds in savory were identified luteolin and naringenin.

5.4.2. Quantitative analysis of polyphenolic compounds

Quantitative analysis of polyphenolic compounds was performed by two methods of analysis: spectrophotometry and high performance liquid chromatography.

5.4.2.1. Spectrophotometric analysis of polyphenolic compounds

By spectrophotometric analysis we determined the total amount of flavonoids and polyphenolcarboxylic acids (caffeic acid type).

5.4.2.1.1. Spectrophotometric analysis of total flavonoids

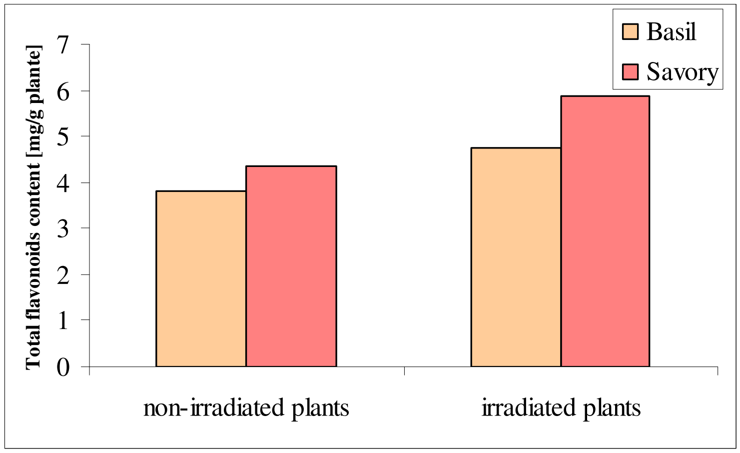


Figure 78. Comparison chart quantity of total flavonoids.

It is noted that the amount of flavonoids in irradiated plants is higher than in the reference plants.

5.4.2.1.2. Spectrophotometric analysis of polyphenolcarboxylic acids

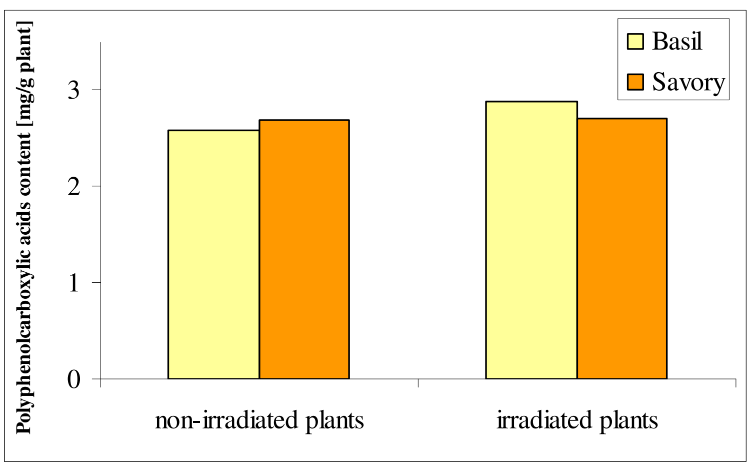


Figure 79. Comparison chart quantity of polyphenolcarboxylic acids type caffeic acid.

The amount of polyphenolcarboxylic acids both in savory as well in the basil increases in irradiated plants compared to the reference.

5.4.2.2. Analysis by high-performance liquid chromatography

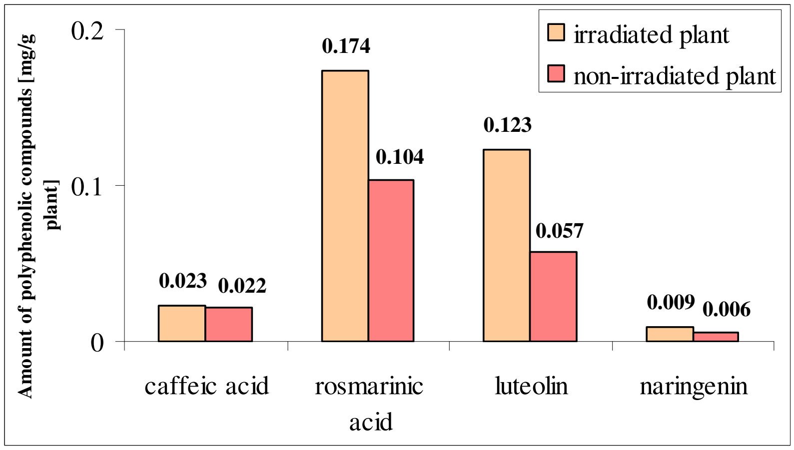


Figure 80. Comparison chart quantity of polyphenolic compounds from *Satureja hortensis* L.

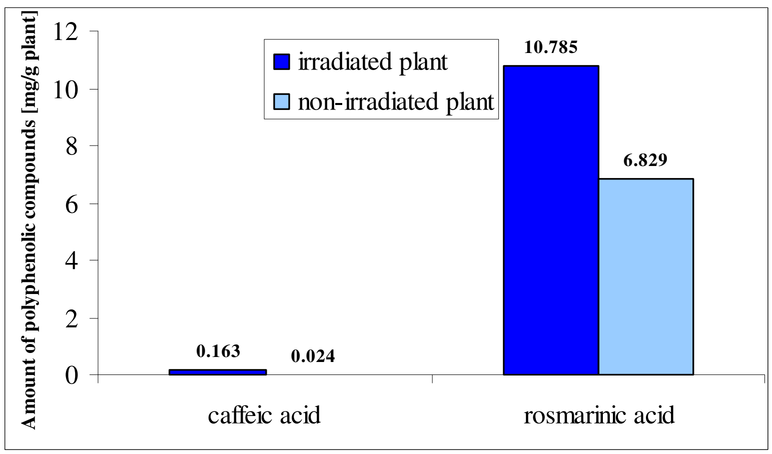


Figure 81. Comparison chart quantity of polyphenolic compounds from *Ocimum basilicum* L.

The major compounds in both savory as well in the basil are rosmarinic acid and caffeic acid. Also, it can be seen that in plants subjected to microwaves irradiation the amount of polyphenolic compounds is higher than in the reference plants.

5.5. Studies on the antioxidand character modification during microwave irradiation plants

The determination of antioxidant activity was performed only on basil because a lot of savory have died before being removed from the experiment chamber.

5.5.1. Sample preparation

In order to study the antioxidant character of the basil, we chose a set of fresh leves, both irradiated and non-irradiated. The leves were gathered from the same set of plants which were used for the polyphenol determination.

One gram of fresh leaves was mortared with 1 g quartz sand after which 25 mL of 80% ethanol is added. The mixture was stirred for 1 h at 4°C and then centrifuged at 10,000 rpm for 15 minutes. The supernatant was removed and the pellet was resumed with 5 mL 80% ethanol, stirred for 15 minutes and centrifuged following the same procedure. Supernatants have been finally united. Each sample was extracted independently in triplicate and the analyzes were performed in the same day.

5.5.2. Determination of antioxidant character of irradiated plands and reference

5.5.2.1. DPPH method

The DPPH method is frequently used due to its stability, simplicity and reproducibility [213]. The antioxidant activity determined by DPPH test for irradiated basil is different from that of the reference which have a lower antioxidant activity.

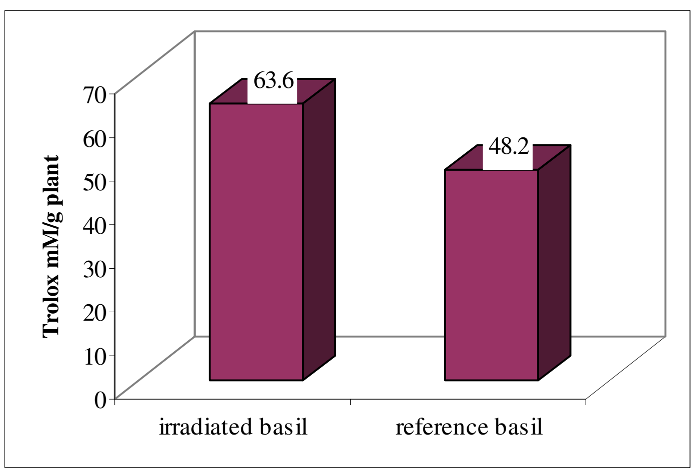


Figure 83. The antioxidant activity of alcoholic extracts analyzed by DPPH method.

5.5.2.2. ORAC method

It was used the method described by Huang et al [215]. ORAC values were calculated as described by Cao and Prior [216].

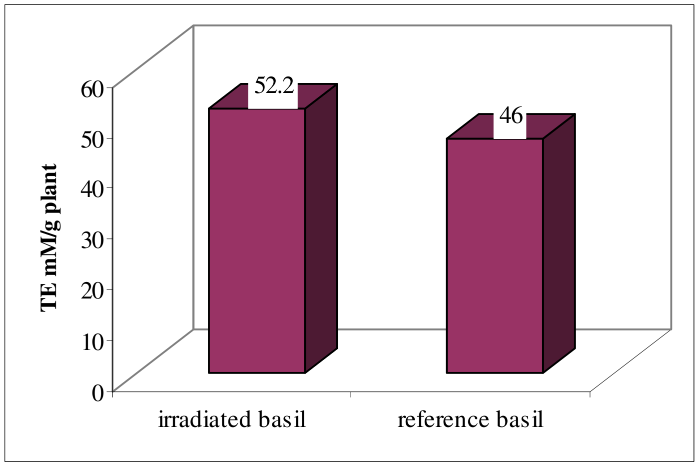


Figure 85. The antioxidant activity of alcoholic extracts analyzed by ORAC method.

Irradiated basil extract (52.2 mM TE/g plant) has a higher ORAC value compared to reference basil extract (46 mM TE /g).

5.5.2.3. TROLOX method

For ABTS assay, the protocol follows the method proposed by Arnao [212].

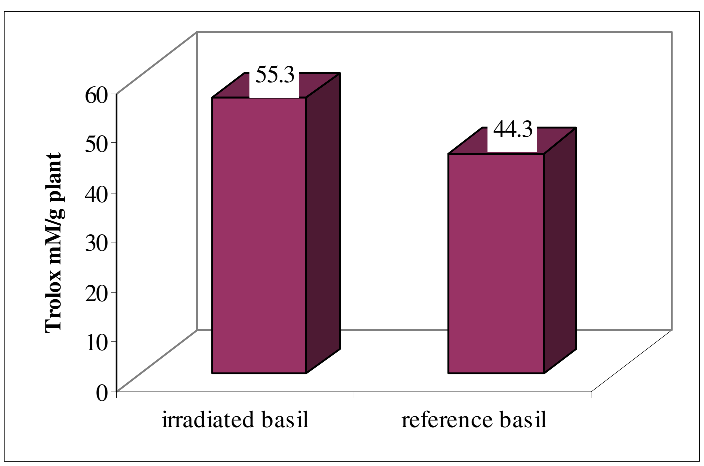


Figure 87. The antioxidant activity of alcoholic extracts analyzed by TEAC method.

Irradiated basil extract has a significantly higher antioxidant activity compared to TEAC value obtained for reference basil extract.

Conclusions

The PhD thesis had as the main objective the determination of microwaves effect on bioactive compounds present in: Satureja hortensis L. (garden savory) and Ocimum basilicum L. (basil). Following the researches have been found that:

- The composition of extracts obtained by maceration, refluxing, ultrasound or in microwave field not vary from qualitative point of view with extraction technique used. The quantity extracted depends on extraction solvent used, the type of bioactive compound extracted and the extraction technique used.
- Efficient extraction conditions of volatile oils from savory and basil, using GC-FID and TLC techniques are: ultrasound in hexane - ethyl ether (1:1, v/v) for savory and extraction in microwave field in diethyl ether - ethanol (1:1, v/v) for basil. These conditions were further used for analysis of plants irradiated and non-irradiated with microwaves.
- The optimal extraction method of polyphenolic compounds (flavonoids and polyphenolcarboxylic acids) is ultrasound for 30 minutes with a mixture of ethanol - water (80:20, v / v) for savory and ethanol - water (60:40, v / v) for basil, method which was later used for the extraction of polyphenolic compounds from irradiated and non-irradiated plants .
- Modification of growing conditions has influenced both development of plants as well the composition of biological active compounds followed in this study.
- Regarding the development of plants were observed as follows: similar development irradiated compared with those non-irradiated (reference) plants in first two months after irradiation; after two months from irradiation the difference between irradiated and non-irradiated plants became visible. Irradiated plants have developed more powerful, were more of green, flourished soon as 3 weeks and tended to ramification much more than the reference plants .
- The content of chlorophylls a and b as well the total content of carotenoids from plants subjected to microwave was smaller than in the reference plants. From the chromatograms obtained by HPLC is not observed significant changes from qualitative point of view, in basil extract irradiated, only β-cryptoxanthin disappears or is only in traces.

- Analyzing the essential oils extracts it was found that thymol and carvacrol are the major components of savoryand in basil was found a large amount of terpenes, sesquiterpenes and diterpenes. This has been observed by TLC, GC-FID and confirmed by GC-MS, existing a good correlation between the three analysis techniques.
- TLC method has established chromatographic fingerprint of savory and basil, both irradiated and non-irradiated (reference) and has led to the following conclusions: after irradiation not appear new bioactive compounds; typically irradiated plants have a higher content of bioactive compounds; the amount of essential oils depends on the stage of maturity of plant (group 2 has a stronger fingerprint than group 1),in the case early stages of development cases is appropriate to follow essential oils precursors.
- GC-MS, like other analysis methods used showed that in irradiated plants bioactive compounds content is greater than in the from the reference. Thus, there is a good correlation between those three methods. In savory have been found as major components thymol and carvacrol, and in basil has been found a large amount of terpenes, sesquiterpenes and diterpenes.
- The amount of essential oils differ and depending on the development stage of plant (basil).
- In irradiated plant total amount of flavonoid and polyphenolcarboxylic acids (caffeic acid type) determined spectrophotometrically is greater than from the reference plant. It was confirmed by HPLC. Therefore, the amount of flavonoids, respectively polyphenolcarboxylic acids in irradiated savory is 35.7%, 0.89% respectively greater than in savory the reference, while in irradiated basil is 24.3%, 11.59% respectively greater than from the reference basil.
- Quantity of polyphenol compounds identified by HPLC (caffeic acid, rosmarinic acid, luteolin, naringenin and apigenin) in extracts obtained from irradiated savory and basil is greater than from the reference plants.
- Obtained extract from irradiated basil has a higher antioxidant activity than the reference plant extract, as evidenced by the three methods of determination used: DPPH, TEAC, ORAC.
- It was observed a strong correlation between changes of antioxidant character and polyphenol content of studied plant extracts, both registered an increase in microwave irradiated plants.

REFERENCES

1. L.M. Penafiel, T. Litovitz, D. Krause, A. Desta, J.M.L. Mullins, *Bioelectromag.*, 18, **1997**, 132-141.

2. T.A. Litovitz, L.M. Penafield, J.M. Farrel, D. Krause, R.Meister, J.M. Mullins, *Bioelectromag.*, 18, **1997**, 422-430.

3. G.J. Hyland, *Eng. Sci. Educ. J.*, 7(6), **1998**, 261-269.

186. E. Surducan, V. Surducan, *Romanian Patent*, RO- 122063, **2008**.

187. M.L. Soran, C. Varodi, S.C. Cobzac, I. Lung, *J. Essent. Oil Bear. Pl.,* 14(6), **2011,** 699-707**.**

188. M.L. Soran, S.C. Cobzac, C. Varodi, I. Lung, E. Surducan, V. Surducan, *Studia UBB Chemia*, LV(3), **2010**, 31-38.

189. M.L. Soran, S.C. Cobzac, C. Varodi, I. Lung, E. Surducan, V. Surducan, *JPCS,* 182, **2009**, 012016.

205. I. Broş, M.L. Soran, R.D. Briciu, S.C. Cobzac, *J. Planar Chromatogr. – Mod. TLC*, 22(1), **2009**, 25-28.

206. XXX *Farmacopea Română* X, Ed. Medicală, Bucureşti, **1993**.

207. XXX *Farmacopea Română* IX, Ed. Medicală, Bucureşti, **1976.**

209. E. Surducan, V. Surducan, A. Halmagyi, *Romanian Patent*, RO 125068/28.02.**2012**.

210. G. Britton, S. Liaaen-Jensen, H. Pfander, " *Carotenoids*", *Volume 1A: Isolation and Analysis*, Birkhäuser Verlag, Basel, Boston, Berlin, **1995**.

212. M. B. Arnao, A. Cano, J. F. Alcolea, M. Acosta, *Phytochem Anal.*, 12(2), **2001**, 138-143.

213. T. Katsube, H. Tabata, Y. Ohta, Y. Yamasaki, E. Anuurad, K. Shiwaku, Y. Yamane, *J. Agric. Food Chem.,* 52(8), **2004**, 2391-2396.

215. D. Huang, B. Ou, M. Hampsch-Woodill, J. A. Flanagan, R. L. Prior, *J. Agric. Food Chem.*, 50, **2002**, 4437-4444.

216. G. Cao, R. L. Prior, *Methods Enzymol.*, 299, **1999**, 50-62.

PAPERS PUBLISHED ON THE TEMATICS OF PHD

A. Papers published on the tematics of PhD

1. Effect of microwave irradiation on polyphenolic compounds from *Satureja hortensis* L.
   I. Lung, M.L. Soran, C. Tudoran, C. Măruțoiu
   Central European Journal of Chemistry (accepted)
2. Essentials Oils Determination from *Satureja hortensis* L. by Chromatographic Techniques
   M.L. Soran, C. Varodi, S.C. Cobzac, I. Lung
   Journal of Essential Oil – Bearing Plants, 14(6), **2011**, 699-707.
3. HPTLC Analysis of Thymol in Extracts of *Satureja hortensis* L. Obtained by Different Techniques
   M.L. Soran, I. Lung
   Journal of Planar Chromatography – Mod. TLC, 23(5), **2010**, 320-322.
4. Extraction and chromatographic determination of essentials oils from *Ocimum basilicum* L. leaves
   M.L. Soran, S.C. Cobzac, C. Varodi, I. Lung, E. Surducan, V. Surducan
   Studia Universitatis "Babes-Bolyai", Chemia, LV(3), **2010**,31-38.
5. HPTLC Quantification of Some Flavonoids in Extracts of *Satureja hortensis* L. Obtained by Use of Different Techniques
   I. Broş, M.L. Soran, R.D. Briciu, S.C. Cobzac
   Journal of Planar Chromatography – Mod. TLC, 22(1), **2009**, 25-28.
6. The extraction and chromatographic determination of the essentials oils from *Ocimum basilicum* L. by different techniques
   M.L. Soran, S.C. Cobzac, C. Varodi, I. Lung, E. Surducan, V. Surducan
   Journal of Physics: Conference Series, 182, **2009**, 012016

C. Papers published in the conference summary, national and international symposiums (selection)

1. Determination of phenolic compounds from *Ocimum basilicum* L. by different extraction methods
   I. Lung, M.L. Soran, M. Stan, E. Surducan, C. Măruțoiu
   Second International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences IC-ANMBES, **2012**, 24 – 27 May, Braşov, Romania
2. Change the antioxidant character of basil by growth in microwave field
   I. Lung, M.L. Soran, C. Matea, C. Bele
   International Conference "Processes in Isotopes and Molecules" PIM, **2011** 29 September – 01 Octomber, Cluj-Napoca, Romania
3. Essentials Oils Determination from *Satureja Hortensis L.* By Chromatographic Techniques
   I. Lung, M.L. Soran, S.C. Cobzac
   34th International Symposium on High-Performance Liquid Phase Separations and Related Techniques, **2009**, 28 June – 2 July, Dresda, Germany
4. HPLC Analysis of flavonoids from *Satureja Hortensis* L.
   I. Broş, M.L. Soran, C. Bele, E. Surducan, V. Surducan, C. Tudoran
   4th meeting on CHEMISTRY AND LIFE, **2008**, 9-11 September, Brno, Czech
5. Different extraction techniques of flavonoid bioactive compounds from *Satureja hortensis* L. and HPTLC analysis
   I. Broş, M.L. Soran, I. Spinu, S.C. Cobzac
   International Symposium for High Performance Thin-Layer Chromatography, **2008**, 11-13 June, Helsinki, Finland