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Extracción y separación de compuestos orgánicos de *Thymus Vulgaris*

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TÍTULO: **Extraction and separation of organic compounds of *Thymus vulgaris* drug and evaluation of the extraction procedures to maximize the concentration of elements in base of needs**

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Extraction and separation of organic compounds of *Thymus vulgaris* drug and evaluation of the extraction procedures to maximize the concentration of elements in base of needs

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Resumen

El objetivo del estudio es la extracción y separación de la planta *Thymus Vulgaris* (tomillo), y la evaluación de los procedimientos de extracción para maximizar las concentraciones en base a las necesidades.

Los métodos de extracción utilizados han sido el método Soxhlet y la maceración dinámica, utilizando como disolventes 96% etanol, etil-acetato y n-pentano para el primer caso; y 96% etanol, mezcla 50:50 etanol absoluto-agua y agua para el segundo caso. Todos los experimentos han sido realizados con la planta original y un residuo de esta obtenido a partir de una destilación de vapor a partir de la planta original.

Con los extractos obtenidos en los métodos anteriores, se realizó un análisis de los compuestos orgánicos utilizando espectrofotometría como método (y un espectrofotómetro como aparato) , en particular de flavonoides, taninos, polifenoles y antioxidantes, realizando las correspondientes muestras para cada compuesto.

Los resultados obtenidos son expuestos en este proyecto.

Palabras clave

Thymus, Soxhlet, maceración, disolvente, extracto

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1.Abstract

The aim is to study the extraction and separation of *Thymus vulgaris* drug and evaluate the procedures of extraction to maximize the concentrations in base of the necessity.

Extracts with different compositions have been obtained from *Thymus Vulgaris* (flavonoids, tannins, polyphenols and antioxidants) applying different techniques. Soxhlet extraction was performed using 96% ethanol, ethyl-acetate and *n*-pentane. Also, maceration extraction was performed using 96% ethanol, ethanol-water 50:50, and water. All experiments were doubled, being done with the original and with the residue of a steam distillation, with the purpose of notice the difference between them. The best extract yield by Soxhlet extraction was obtained with 96% ethanol, whose yield was ***27,3 ± 3,3%.*** The best extraction yield in the maceration was obtained using water like solvent with a yield of ***31,19%.***

After that, with the extracts of Soxhlet and maceration methods, analysis of organics compounds were done. In view of the results, it is possible to conclude that in the thymus the flavonoids percentage is very low with each solvent, being the highest the Soxhlet with 96% ethanol **2,672** ***g quercetin equivalent/100g extract.*** In the case of polyphenols and tannins, the method which provides more yield is the maceration with the ethanol-water 50:50 solvents; **28,389** ***g pyrogallol equivalent/ 100g extract*** for the polyphenols, and **11,691** ***g pyrogallol equivalent/ 100g extract*** for the tannins, being this last result obtained with the residue. Referring to antioxidants, the result best obtained was in the 96% ethanol **(15,23 µg/ml).**

2. Introduction

Thymus vulgaris (Thyme as common name) is a plant which is normally used in cooking, but also as ingredient in natural remedies to relieve symptoms of slight and common soreness. Although is used as condiment or herb to prepare infusions, it can be also used as essential oil. [1]

The reason of the benefits of the *Thymus* is a large amount of bioactive compounds. The most relevant characteristic of the *Thymus* is his antioxidant capacity, containing also flavonoids and polyphenols. [2]

The main goal of this work is to extract and find out the best solvent and method using different solvents of non-identical polarity and extractions in series, and different methods based on the kind of boiling.

The organic solvents used were 96% ethanol, mixtures 50:50 (v/v) of ethanol-water and water for the maceration . Also 96% ethanol, pentane and ethyl-acetate for the Soxhlet method. The extraction were applied to pure milled *Thymus Vulgaris* and the residue of it after a steam distillation.

The moisture content and particle size distribution of the milled *Thymus vulgaris* were also measured before the extraction.

Spectrophotometer analysis was performed to know the concentration of antioxidants, flavonoids, polyphenols and tannins in the different extracts.

3. Theoretical Background

3.1 Thymus vulgaris

The *Thymus vulgaris* genus, contains around 300 species, better-known as Thyme, common thyme or garden thyme.

The origin of the *Thymus vulgaris* is the south of Europe and Asia. It comes from the order of *Lamiales* and the family of *Lamiaceae*. Thymus can be described like a small bush with a height between 13-40 cm, with the steams erect and woody, and the leaves small with rolled edges and wrinkled; the flowers are small pink and the odor is aromatic. This plant typically grows in the edges of dry roads. [1]

The first application of thymus was the embalmment of mummy in the ancient Egypt. However the firsts in applications in medicine of the thymus was in the ancient Greece as medicinal plant, to cure deep injuries. In the same way the plant was treated int the Middle-Age, being a very valuable plant as medicine against asthma and dyspnea. [2]

Nowadays, thymus is used like source of essential oils, besides as medicine in the folk medicine, but growing up in the traditional medicine. Also is very important in cooking as seasoning, very typical in mediterranean cuisine, due to the intense smell and the characteristically flavor given. At last is also used to make infusions, helping to relieve respiratory problems. [1]

The phytochemistry of Thymus Vulgaris includes a variety of compounds, with antioxidant properties, thanks to the flavonoids content on it, and in a lesser extent, tannins and polyphenols. [3]

3.2 Thymus essential oil

Among the most powerfully cleansing essential oils, this pungent, thyme oil is a core resource of the plant world to purify and enhance immunity. Thymol, the predominant volatile compound in this thyme distillation, possesses warming, stimulating qualities that purify and boost immunity.

Thyme's high thymol content encourages expectoration and its warming effect reduces excesses of mucous and phlegm. When diluted in massage oil, thyme oil can help to enhance circulation and to aid healthy muscle and joint function. Used in the diffuser with other oils such as conifers, thyme oil strengthens the nerves and enhances brain function, improving memory and concentration. [5,6]

The odor of the thymus oil is herbaceous, pungent, slightly sweet, medicinal, spicy, being strong.

In the way of the components of the oil, around 25 are shown in the chromatogram. The three major components of the essential oil of thyme are shown in Table 1.[6]

Table 1. Major components of the essential oil of thyme

No.	*Compound*	*Percentage (%)*
1	**Para-cyrene**	8,41
2	**Gamma-Terpinene**	30,9
3	**Thymol**	47,59

****Results taken from a experiment done in a HPLC chromatogram alien to the thesis.**

3.3.1 Antioxidants

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that produces free radicals, permitting to chain reactions that could damage cells.

To make a balance of the oxidative state, plants and animals have complex systems of overlapping antioxidants, as glutathione and enzymes produced by their owns or the dietary antioxidants, vitamin A, vitamin C, and vitamin E. [7]

Antioxidants are classified in two ways, depending on the solubility in water (hydrophilic) or in lipids (lipophilic). In general, hydrophilic antioxidants react with oxidants in the blood plasma, while lipophilic antioxidants protect cell membranes from lipid per-oxidation.[6]

- **Hydrophilic antioxidants:** vitamin B complex and vitamin C.
- **Lipophilic antioxidants:** vitamin A, vitamin D, vitamin E and vitamin K. [8]

3.2.1 Flavonoids

According to IUPAC, the term " flavonoid" is commonly used to include not only natural products, but also synthetic compounds related to them. The structural feature of this family of compounds is based on derivatives of a phenyl-substituted 1-phenylpropane possessing a C15 skeleton, except for the rotenoids, which have a C16 skeleton but are also phenyl-substituted 1-phenylpropane derivatives, and that of flavonolignans, whose structure is based on flavonoids condensed with C6-C3 lignan precursors. [9]

In the next table the most important flavonoids are shown:

Table 2. Main flavonoids

Flavonoid	Biological activity
β-Carontene	Antioxidant
Lutein	Additive Antioxidant
Quercitin	Therapeutic properties
Cryptoxanthin	Pigment,Provitamin A
Carotene	Photosynthetic pigments Antioxidant
Capsitina	Antioxidant, anti-fungal agent
Hesperidin	Defensive role
Anthocyanins	Pigment
Lycopene	Photo-protection
Catechin	Antioxidant
Resveratrol	Protection against pathogens
Rutoside	Ligand

3.2.2 Polyphenols

Polyphenols are a structural class of mainly natural, but also synthetic or semisynthetic, organic chemicals characterized by the presence of large multiples of phenol structural units. The number and characteristics of these phenol structures underlie the unique physical, chemical, and biological properties of particular members of the class. [10]

Table 3. Groups of polyphenols

Polyphenolic group	Biological activity
Phenolic acids	Organic compound
Stilben	Fungus defend
Lignan	Defense antioxidant
Flavonoids	Pigment, antioxidants

3.2.3 Tannins

A tannin is an polyphenolic biomolecule that precipit proteins and another organic compounds as amino-acids and alkaloids. They are a kind of flavonoid too. [11]

The tannin compounds are widely distributed in many species of plants, where they play a role in protection from predation, and perhaps also as pesticides, and in plant growth regulation. [12]

Tannins have molecular weights ranging from 500 to over 3,000 u (gallic acid esters) and up to 20,000 u.

There are three main classes of tannins, shown in the table 4:

Table 4. Main types of tannins

Base Unit	Class/Polymer	Sources
Gallic acid	Hydrolyzable tannins	Plants
Flavone	Condensed tannins	Plants
Phloroglucinol	Phlorotannins	Brown algae

The normal location of tannin s in plants are leaf, bud, seed, root, and stem tissues.

Also, in reference to the cellular location, tannins are manufactured by a chloroplast-derived organelle, the tannosome. Tannins are mainly physically located in the vacuoles or surface wax of plants. These storage sites keep tannins active against predators, but also keep some tannins from affecting plant metabolism while the plant tissue is alive; it is only after cell breakdown and death that the tannins are active in metabolic effects. [13]

3.3 Plant compounds extraction

Different kind of techniques have been used to extract bioactive products from natural matrices, starting with the traditional ones and including the most recent ones (supercritical fluid extraction, pressurized water extraction….). [14]

Maceration: In this process (used in this work), the whole plant is placed in a stoppered container with the solvent and allowed to stand at bigger temperature for a period of 1 hour. The mixture then is strained, and the combined liquids are clarified by filtration or decantation after standing, following this process for 3 steps. [14]

Digestion: This is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the solvent is thereby increased.

Decoction: In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. The starting ratio of crude drug to water is fixed, the volume is then brought down to one-fourth its original volume boiling it during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further. [14]

Percolation: This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. The solid ingredients are moistened with an appropriate amount of the specified solvent and allowed to stand for approximately 4 hours in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional solvent is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. 14

Hot Continuous Extraction (Soxhlet): In this method, the finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber of the Soxhlet gadget. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent because the solvent is evaporated each cycle. On the other hand the extraction time is long, and the device spends a lot of energy to evaporate solvent. At small scale, it is employed as a semi-batch process only, but it becomes much more

economical and viable when converted into a continuous extraction procedure on medium or large scale.

One of the main aspect to take in account is the selection of the solvent. Depending on the election of the solvent yields and concentration of the extracts will be different. The most important characteristic to take in account at the time to choose a solvent is the polarity of the same.

Aqueous Alcoholic Extraction by Fermentation: Medicinal preparations adopt the technique of fermentation for extracting the active principles. The extraction procedure involve soaking the crude drug, in the form of a powder or a decoction, specifying a period of time, during it is undergoes fermentation and generates alcohol; this facilitates the extraction of the active constituents contained in the plant material. In large-scale manufacture, wooden vats, porcelain jars or metal vessels are used in place of earthen vessels. [14

Counter-current Extraction: In counter-current extraction (CCE), wet raw material is pulverized using toothed disc disintegrators to produce a fine slurry. In this process, the material extracted is moved in a only direction within a cylindrical extractor where it comes in contact with the solvent. The further the starting material moves, the more concentrated the extract becomes. The process is highly efficient, requiring little time and posing no risk from high temperature. Finally, the concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end. [14]

3.4 Chemical analysis

To make the analysis of the experiments, spectrophotometry will be the way to work. Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.

In spectrophotometry, photometers are used, known as spectrophotometers, that can measure a light beam's intensity depending on the wavelengths.

The most common (and the one that concern us), is the Ultraviolet-visible spectroscopy (Uv-Vis). It refers to absorption spectroscopy in the ultraviolet-visible spectral region. The absorption (or reflectance) in this range affects the perceived color of the chemical products involved. [16]

The principle of the method is the next: There are some molecules that contain π-electrons or n-electrons which absorb energy as ultraviolet or visible light, with the objective of excite electrons to higher molecular orbitals. Electrons are easily excited, the longer the wavelength of light it can be absorbed. It is based in four types of transition- π-π*,n-π*,σ-σ*,n-σ*.The requirements of energy for various transitions follow the next order σ-σ*>n-σ*>π-π*>n-π*. [16]

The gadget used to make the measurement is a spectrophotometer. Spectrophotometer is used, once known the wavelength, as link between values of an equal photometric link relatives to two beams of radiation and the concentration measured in the sample. This instrument has the ability to project a monochromatic light beam through a sample and measure the amount of light that is absorbed by said sample.

The spectrophotometer, consists of two devices, spectrometer and a photometer. The spectrometer is the one that produces, scatters and measures light. The photometer has a photoelectric detector that measures the intensity of light.

Spectrometer: Produces a desired range of wavelength of light. First a collimator transmits a straight beam of light (photons) that passes through a monochromator (prism) to divide it into several components of wavelengths (spectrum). Then a wavelength selector transmits only the desired wavelengths.

Photometer: After the chosen wavelength of light passes through the sample solution in the cuvette, the photometer detects the amount of photons being absorbed and then sends a signal to a galvanometer or a digital display.

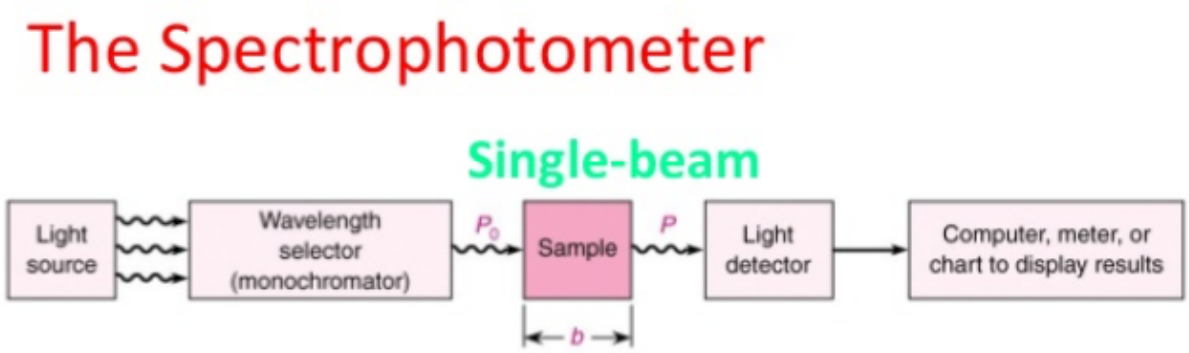


Figure 1: spectrophotometer diagram

Components of a spectrophotometer: [17]

- *Source of light*: The light source that illuminates the sample must meet the following conditions: stability, directionality, distribution of continuous spectral energy and long life. The sources used are: tungsten lamp (also called tungsten), xenon arc lamp, deuterium lamp and LED lamp used in laboratories.

- *Monochromator:* The monochromator isolates the desired wavelength radiation that impinges or is reflected from the set, is used to obtain monochromatic light. It consists of the entrance and exit slits, collimators and the dispersion element. The collimator is located between the entrance and exit slits. It is a lens that carries the light beam entering a certain wavelength into a prism which separates all wavelengths from that beam and the desired length.

- *Sample compartment:* It is where the interaction with matter takes place. It is important to note that during this process the Lambert-Beer law* applies at its maximum expression, based on its absorption laws.

- *Detector*: The detector is responsible for capturing the radiation and, in turn, leave it in evidence for later study. There are two types: the ones that respond to photons, and the ones that respond to heat.

- *Cells*: They are the containers where the liquid samples to be analyzed are deposited. The material of which they are made varies according to the region that is working; Are glass or plastic if the work is with the visible region, quartz if the work is in the ultraviolet and NaCl if the work is in the infrared region. They are characterized by having two walls corresponding to the optical sides through which the beam of light crosses.

4. Materials and Methods

4.1 Materials

4.1.1 The plant. Thymus vulgaris

The plant *Thymus vulgaris* used in the work was received in a 20 kg. big bag, supplied by *Rózsavölgyi Kft.* The plant was milled, and the color was obscure green. The particle size, density and moisture content were measured.

4.1.2 Chemicals

The compounds used during the work to reach the objectives were:

- Ethanol (C_2H_6) supplied by Molar Chemicals Kft. Purity: 99,94%
- Distillate water (H_2O): from the laboratory
- Ethanol 96% (C_2H_6) supplied by Molar Chemicals Kft. Purity: 96,08%
- Methanol (CH_4O) supplied by Molar Chemicals Kft. Purity: 99,99%
- *n*-Pentan (C_5H_{12}) supplied by Molar Chemicals Kft. Purity: 98,03 %
- Ethyl-Acetate ($C_4H_8O_2$) supplied by Molar Chemicals Kft. Purity 99,98 %
- Folin-Ciocalteu's phenol reagent supplied by Merck Kft.
- Sodium Carbonate (Na_2CO_3) supplied by Sigma-Aldrich Co.
- Hide power (from bovine hide) supplied by Sigma-Aldrich Co.
- DPPH or 2,2-Diphenyl-1-pircrylhydrazyl, free radical ($C_{18}H_{12}N_5O_6$) supplied by Sigma-Aldrich Co.

4.2 Methods

4.2.1 Determination of moisture content

The moisture was determined according the gravimetric method.

1. Around 10 grams of thymus was weighted in a Petri glass (M1).

2. The glass is set in the oven around 2 hours with 105ºC degrees of heating.

3.The glass is taken out of the oven and let it warm until room temperature.

4.Weigh the Petri glass (M2).

The calculus of the moisture will follow the next equation:

$$\%Moisture = \frac{M1 - M2}{M1} * 100$$

Equation (I)

Being M1 the weight of the plant before heating in the oven and M2 the weight after that.

4.2.2 Determination of particle size distribution

With the objective of know the particle size distribution, the particles have been separated in different size fractions, and after that it has been determined by a statistic method (Rossin-Ramler-Sperling-Bennet-RRSB).

1. A good election of the configuration of the sieves that are going to be used must be done.

2. The sieves are weighted before the sieving.

3.The sieves are colocated in the decreasing order and fix them in the sifter.

4. 60 grams of Thymus are set on the top of the sieves.

5. Sieve during 20 minutes at 40 Hz.

6. Each sieve is weighted.

7. Using the STADISTICA program, the mean particle size and the uniformity factor (n) are calculated.

The experiment has been repeated three times, calculating the average.

4.2.3 Determination of Thymus vulgaris density

The circuit compounds next gadgets: One empty bottle in a rack, one plug for the bottle (which is closed and opened to fixer necessities) connected with the water circuit, a faucet to increase or reduce the water level, and a metrical system to find out the highness. There will be two lines; the lowest will be used with the system opened, and the highest (Vm) which will show the highness of the experiment with the closed circuit.

1. Bubbles in the circuit must be thrown off.

2. The empty bottle must be put in his position and close it.

3. Level the first line and close the system, closing the plug of the bottle.

4. The gadget will reach Vm, increasing the highness each time.

5. Te bottle is weighted, as many times as the flask is full of the plant.

The experiment has been done three times, calculating the average.

4.2.4 Extractions

4.2.4.1 Soxhlet extraction

The soxhlet extractor used during the experiments where located in the DCs building, first floor. Laboratory Soxhlet ia an appliance which permits the performance of extractions on a small scale in a semi batch way. The Soxhlet parts compounds:

- Heater: Gives to the solvent heat enough to the evaporation.
- Boiling flask: Glass where the solvent is located to evaporate, and are collected the previous extracts.
- Thimble: Filter made of paperboard which contains the plant.

- Extraction chamber:Glass which contains the tumble, and where the solvent and the raw keep on touch.
- Siphon: Gadget that determines the maximum level of solvent in the chamber before the reflux.
- Condenser: Jacketed arm (with water), where the evaporated solvent is cooled and becomes liquid again, coming back to the chamber.

The next figure shows a Soxhlet extractor:

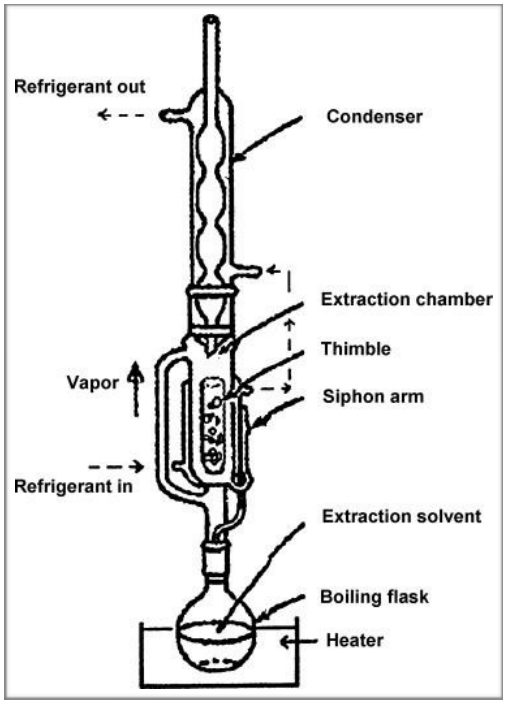


Figure 2: Soxhlet diagram

1. The thimble is filled three quarters. It was, more or less, 20 grams of plant.
2. The thimble is introduced in the extraction chamber.
3. The chamber is spliced to the condenser.
4. An empty distillation flask is weighted.
5. The distillation flask is filled with 200 ml of solvent.

6. The flask is fixed to the extraction chamber, tweezing it.

7. Switch on the cooling water in that going to pass through the jacket.

8. Switch on the thermostatic bath, fixing the set up heating power.

9. Let the process work until the solvent refluxed becomes transparent.

10. Switch off the thermostatic bath while the solvent is leaving through the siphon.

11. Wait a while (10 minutes) to switch off the cooling water.

12. Disassemble the gadget.

13. Evaporate the solvent in the flask using the rotatory distillator.

    13.1 Fix the false to the vacuum evaporator.

    13.2 Switch on the cooling water to condensate.

    13.3 Switch on the thermostatic bath and fit the set up heating power.

    13.4 Once reached the temperature desired, switch on the vacuum pump and the valve.

    13.5 Regulate the vacuum until the liquid had disappeared from the flask.

    13.6 Switch off the vacuum.

    13.7 Disassemble the evaporator.

    13.8 Let the flask cool down until room temperature.

    13.9 Weigh the distillation flask (Ma).

    13.10 Scratch the extract from the walls of the flask and keep it in a vial.

14. Leave the thimble drying at room conditions during a couple of days.

15. Pour all the residue inside the thimble in a storage glass.

Three test are extracted at the same time to do an average.

The equation needed to calculate the yield (Y) of the extraction is the next:

$$Y(\%) = \frac{Mb - Ma}{Mt} * 100$$ ***Equation (II)***

Where Mb is the weight of the empty flask, Ma is the weight of the flask after the extraction with the extract inside, and Mt is the weight of dry material into the thimble.

4.2.4.2 Dynamic maceration

The dynamic maceration is a kind of extraction used in the work, located in the first floor of DCs. The steps to make a dynamic maceration are:

1. Put into a flask 10 grams of the raw mixed with 200 ml of the solvent.
2. Put the flask in the mixer, switching on the rotation of the flask and the thermodynamic bath.
3. Wait one hour since the beginning of the rotation.
4. Switch off the thermodynamic bath and the rotation, waiting for a while until the flask reach the room temperature.
5. Filter the solution, separating the solvent and the raw.
6. Evaporate the solvent in the flask using the rotatory distillator.
    - Fix the false to the vacuum evaporator.
    - Switch on the cooling water to condensate.
    - Switch on the thermostatic bath and fit the set up heating power.
    - Once reached the temperature desired, switch on the vacuum pump and the valve.
    - Regulate the vacuum until the liquid had disappeared from the flask.
    - Switch off the vacuum.
    - Disassemble the evaporator.
    - Let the flask cool down until room temperature.

- Weigh the distillation flask (Ma).
- Scratch the extract from the walls of the flask and keep it in a vial.

7. The raw is introduced again in the flask, and is faded with another 200 ml again.
8. Repeat again all the steps.

The maceration will be done three times. To calculate the yield, we use the equation 2, only changing that the final yield is the summitry.

$$Y(\%) = \sum \frac{Mb - Ma}{Mt} * 100$$ ***Equation (III)***

4.2.4.3 Steam distillation

Steam distillation is a distillation which permit extract the essential oil from the plant. The steps followed to realize it are:

1. 50 grams of the plant are weighed and introduced it in a big flask.
2. Half liter of distilled must be introduced in the flask with the thymus, and introduce it in the thermodynamic bath.
3. Distilled water will be introduced by the underside of the circuit, until the pipe reach the outfall. This amount of water must not have bubbles.
4. The heating must be put at 150ºC, and wait 4 hours to the finish of the experiment, watching eventually the good working of the process.
5. Put off the circuit and the essential oil is obtained.
6. The volume of the essential oil obtained must be written.
7. Make the yield calculations of the oil obtained.

**As a note, the pipes of the circuit must be covered with aluminum paper, trying to avoid the lost of heat.

4.2.5 Chemical analysis: Spectrophotometer

4.2.5.1 Spectrophotometer sample preparation

The samples are going to be prepared with the objective of find out if the plant has polyphenols, tannins, antioxidants and flavonoids, and the quantity of them. The way to prepare the sample of each one is different.

- ***Polyphenols***

  1. A solution from the extract must be done, with a 0,5-2,5 mg/ml concentration with 96% ethanol in the ultrasound bath.

  2. In a dark volumetric flask of 20 ml measure put:

     - 800 µl of the sample solution
     - 4 ml of distilled water
     - 400 µl Folin-Ciocalteu reagent
     - Na_2CO_3 until reach the line of the flask

  3. Homogenize with the vortex.

  4. Wait 30 minutes.

  5. The absorbance at 760 nm is measured.

  6. The blind sample must be distilled water.

- ***Tannins***

  1. A solution from the extract is done, with a 0,5-2,5 mg/ml concentration with 96% ethanol in the ultrasound bath.

  2. 10 ml of the sample solution are measured with 100 mg of hide power.

  3. Leave it in the ultrasound bath 1 hour.

  4. Filter the solution.

5. In a dark volumetric flask of 20 ml measure put:

- 800 µl of the sample solution
- 4 ml of distilled water
- 400 µl Folin-Ciocalteu reagent
- Na_2CO_3 until reach the line of the flask

6. Homogenize with the vortex.

7. Wait 30 minutes.

8. The absorbance at 760 nm is measured.

9. The blind sample must be distilled water.

- ***<u>Flavonoids</u>***

1. A solution from the extract is done, with a 0,5-2,5 mg/ml concentration with 96% ethanol in the ultrasound bath.

2. In a cuvette, measure:

- 0,5 ml of the sample solution
- 1 ml of $AlCl_3$ (2%)
- 1,5 ml 96% ethanol

3. Homogenize with the vortex.

4. Wait 15 minutes with the cuvettes kept in a dark place.

5. The absorbance at 430 nm is measured.

6. The blind sample must be 0,5 ml of sample solution added to 2,5 ml 96% ethanol.

- ***Antioxidants***

1. 20 mg of DPPH are put in a dark flask of 50 ml, solving it with 50 ml of methanol. At the moment of the measure, a part must be diluted with methanol to reach and absorbance between 0,7 and 0,9.

2. Prepare the sample solution solving 10 mg of extract in 20 ml of methanol.

3. Use methanol as blind.

4. Put un each cuvette 2,5 ml of the lowered DPPH, and put in each cuvette an increasing volume of sample solution. (For example 0,50,100,200 (µl)…).

5. Each cuvette must be homogenized in the vortex and wait exactly half hour. The cuvettes should be protected from the light, so it will be covered all the cuvettes with aluminum paper.

6. Measure the absorbance in 517 nm.

*During the measurement, A_0 absorbance is the one of the DPPH without any sample solution, while A1 absorbance is the one of the absorbance in the first cuvette. Inhibition for each cuvette can be determined with this equation:

$$Inhibition(\%) = \frac{A_0 - A_1}{A_0} * 100$$ ***Equation IV***

The antioxidant efficiency of the extract is compared at 50% inhibition, which is measured from the curve, made from the datas. IC50 is the concentration, where the radicals are been reduced to the half.

4.2.5.2. Spectrophotometer procedure

Spectrophotometer is located in the Das building, in the first floor. The device installed is compounded by:

Once that the samples are prepared, the steps to follow are:

1. Switch on the interruptor in the back of the gadget.

2. Wait 15 minutes necessaries to the spectrophotometer to do the warm up.

3. Switch off the D2 lamp (it's not necessary to make our analysis).

4. Fix the wavelength correct to work.

5. Introduce the blind in the first position of the box, and in the rest of wholes introduce samples.

6. Choose the option 1, "Basic"

7. Pull out the bottom from the blind's position, appearing in the screen the absorbance.

8. Do it as many as samples are prepared.

5. Results

5.1 Moisture

The moisture content of the *Thymus vulgaris* has been calculated following the method explained two chapters above:

Table 5.1.1: Dry content for the pure raw

	Measurement 1	Measurement 2	Measurement 3
Mf (g)	103,93	96,54	86,6
Ma (g)	115,52	106,78	96,93
Md (g)	11,59	10,24	10,33
Mu (g)	114,496	105,819	96
Mw (g)	1,02	0,96	0,93
Moisture (%)	8,84	9,38	9,00

Where Mf is the mass of the empty flask, Ma is the mass of the wet Thymus plus the mass of the empty flask, Md is the mass of dry Thymus in the flask, Mu is the mass of wet Thymus to dry, Mw is the mass of water.

In view of the results (all of the work in the same way), the average moisture content of the *Thymus vulgaris* is: 9.07 ± **0.3 %.**

In the next table the dry content of the residue.

Table 5.1.2: Dry content for the residue

	Measurement 1	Measurement 2	Measurement 3
Mf (g)	103,9	96,53	86,64
Ma (g)	119,89	107,98	108,19
Md (g)	15,99	11,45	21,55
Mu (g)	117,67	106,52	104,7
Mw (g)	2,22	1,46	3,49
Moisture (%)	13,88	12,75	16,19

In view of the results (all of the work in the same way), the average moisture content of the *Thymus vulgaris* is: 14,28 ± **1,75 %.**

This value will be used in further sections to calculate the weight of dry raw. The yields have been calculated in relation to the dry raw, making possible to compare it. The equation to find out the moisture is the next.:

$$Moisture(\%) = \frac{Ma - Mu}{Ma - Mf} x100$$ ***Equation IV***

5.2 Particle size distribution

The mass distribution in the sieves was introduced in the STATISTICA program for each test, and the program calculates and shows the value of the particle size (x0) and the uniformity factor (n). The results are shown in the table below:

Table 5.2 Particle size distribution experiments.

Experiment	x0 (mm)	n
1	0,78	1,654
2	0,769	1,724
3	0,766	1,688

According to the results of the individual experiments the average particle size for this *Thymus Vulgaris* is: 0.772 ± 0.0074 mm. The result for the uniformity factor is 1.689±0,035.

That means that the results are reasonable uniforms, and the particles are quite similar and uniform.

The calculations are shown in the appendix.

5.3 Obtention of density

Three measurements were done with the original Thymus in the densimeter to find out the thymus density.

The density of the original *Thymus vulgaris* is 1256,12 kg/m3.

The density of the residue of *Thymus vulgaris* is 1157 kg/m3.

The difference of density can be explained by the normal error happened during the test done. However, the difference is quite small, so it is possible that the density is similar between both materials.

The calculations are shown in the appendix.

5.4 Soxhlet extraction yields

Two kinds of raw are going to be used. The first one is going to be the "original". It means that the plant never has been used before. The second one is the residue of the *thymus*. It means that in the pilot plant a steam distillation has been done, and the *thymus* residue was utilized. The essential oil of the thymus was obtained.

The Soxhlet method at laboratory scale was used to obtain the extract of the *Thymus* and compare the possibilities of the solvents with another polarities on the original *Thymus*, as well as the extraction yield and selectivity obtained in the performance Soxhlet extraction over the residue. The results obtained for different solvents and raw material are shown in the following tables:

In the next table it is possible to see the results of Soxhlet extraction of pure Thymus Vulgaris with 96% ethanol.

Table 5.4.1 Soxhlet extraction results for 96% ethanol.

96% Ethanol	Sample 1	Sample 2	Sample 3
Dry Thymus	10,36	9,69	9,84
Empty flask	116,25	120,92	96,01
Flask + extract	118,8	123,92	98,61
Extract	2,55	3,00	2,60
Residue	7,81	6,69	7,24
Yield	24,61	30,96	26,42

In relation to the results of each experiment, the average yield for Soxhlet extraction of the original *Thymus Vulgaris* with 96% ethanol is: ***27,3 ± 3,3%***

The temperature of the oil bath was 140ºC-150ºC in semi-batch, being the extraction temperature below the boiling point of the solvent at atmospheric pressure and the vacuum temperature was around 40ºC, being the pressure below 400 mBar.

In the next table results of original Thymus with Pentane are presented:

Table 5.4.2 Soxhlet extraction results for Pentane.

Pentane	Sample 1	Sample 2	Sample 3
Dry Thymus	10,14	10,49	10,15
Empty flask	116,05	113,48	129,01
Flask + extract	116,54	114,22	129,6
Extract	0,49	0,74	0,59
Residue	9,65	9,75	9,56
Yield	4,83	7,05	5,81

In relation to the results of each experiment, the average yield for Soxhlet extraction of the original *Thymus Vulgaris* with Pentane is: **5,9 ± 1,11%**

The temperature of the oil bath was 70ºC in semi-batch, and the vacuum temperature was room temperature, being the pressure below 400 mBar.

In the next table results of original Thymus with Ethyl-Acetate are presented:

Table 5.4.3 Soxhlet extraction results for Ethyl-Acetate.

Ethyl-Acetate	Sample 1	Sample 2	Sample 3
Dry Thymus	9,01	10,5	9,75
Empty flask	96,06	104,53	111,11
Flask + extract	97,29	105,9	112,34
Extract	1,23	1,37	1,23
Residue	7,78	9,13	8,52
Yield	13,65	13,05	12,62

In relation to the results of each experiment, the average yield for Soxhlet extraction of the original *Thymus Vulgaris* with Ethyl-Acetate is: **13,11±0,52%**

The temperature of the oil bath was 70ºC in semi-batch, and the vacuum temperature was room temperature, being the pressure below 400 mBar.

Table 5.4.4 Soxhlet extraction results for 96% ethanol for the residue.

96% Ethanol Residue	Sample 1	Sample 2	Sample 3
Dry Thymus	8,58714	8,86138	8,75854
Empty flask	113,48	122,32	104,48
Flask + extract	115,89	124,58	107,06
Extract	2,41	2,26	2,58
Residue	6,17714	6,60138	6,17854
Yield (%)	28,07	25,50	29,46

In relation to the results of each experiment, the average yield for Soxhlet extraction of the original *Thymus Vulgaris* with 96% ethanol is: **27,68 ± 2,01**

The temperature of the oil bath was 140ºC in semi-batch, and the vacuum temperature was room temperature, being the pressure below 400 mBar.

Table 5.4.5 Soxhlet extraction results for *n*-pentane for the residue.

***n*-Pentane Residue**	**Sample 1**	**Sample 2**	**Sample 3**
Dry Thymus	9,37558	9,3413	9,87264
Empty flask	115,79	96,04	116,27
Flask + extract	116,02	96,31	116,77
Extract	0,23	0,27	0,50
Residue	9,15	9,07	9,37
Yield	2,45	2,89	5,06

In relation to the results of each experiment, the average yield for Soxhlet extraction of the original *Thymus Vulgaris* with Pentan is: **3,47 ± 1,4%**

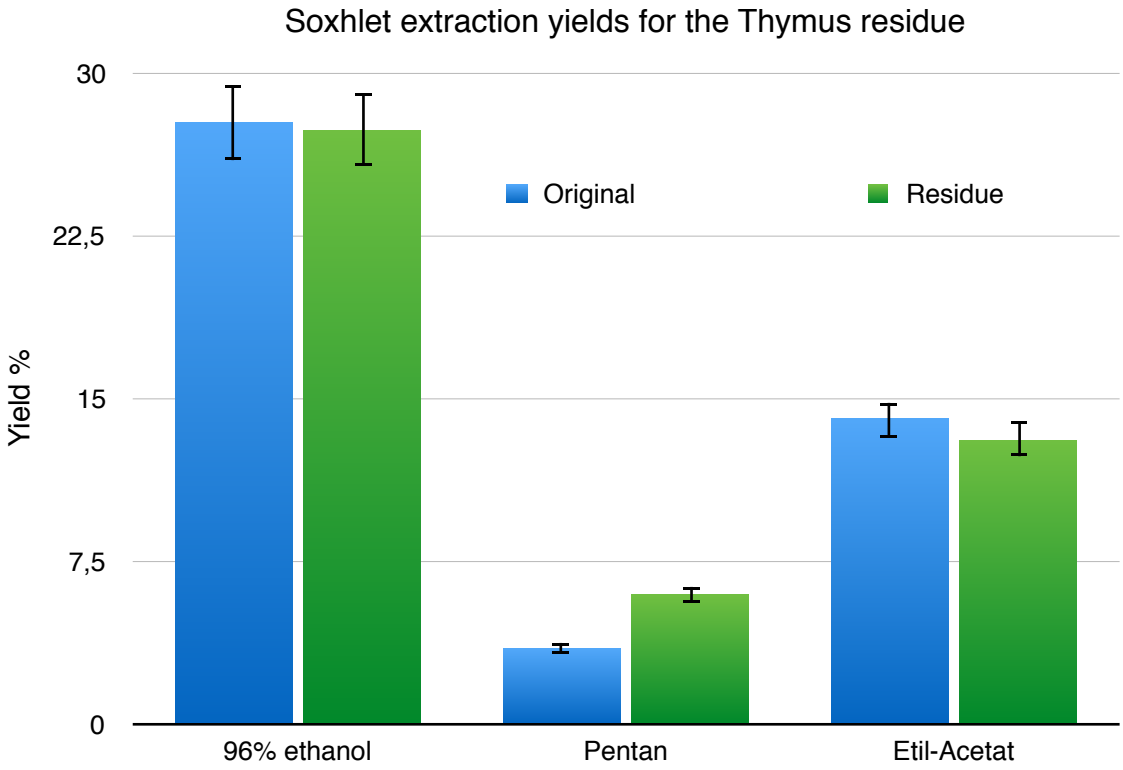
The temperature of the oil bath was 70ºC in semi-batch, and the vacuum temperature was room temperature, being the pressure below 400 mBar.

Table 5.4.6 Soxhlet extraction results for the residue with ethyl-acetate.

Ethyl Acetate Residue	**Sample 1**	**Sample 2**	**Sample 3**
Dry Thymus	9,52984	8,71569	9,17847
Empty flask	116,02	121,05	122,38
Flask + extract	117,52	122,3	123,47
Extract	1,5	1,25	1,09
Residue	8,02984	7,46569	8,08847
Yield	15,74	14,34	11,88

In relation to the results of each experiment, the average yield for Soxhlet extraction of the original *Thymus Vulgaris* with ethyl-acetate is: **13,99 ± 1,95**

The temperature of the Soxhlet was 130ºC in semi-batch, and the vacuum temperature was room temperature, being the pressure below 400 mBar.



It is observed that the solvent whose yield is the highest on the original raw material is the 96% ethanol, this is due to the higher polarity of ethanol respect the pentane and the ethyl-acetate. The mixture of compounds with different polarities helps to make an extraction with better results.

In the case of the residue, the explanation is the same, and the 1-2% lower about the original one can be explained with experimental error, although it also could be because with original raw, essential oil is extracted from the soxhlet, and the properties can be a little bit higher than the residue.

5.5 Dynamic maceration

As in the Soxhlet method, original and residue raw will be used.

The Dynamic Maceration method at laboratory scale was used to obtain the extract of the *Thymus* and compare the possibilities of the solvents with another polarities on the original *Thymus*, as well as the extraction yield and selectivity obtained in the performance Soxhlet extraction over the residue. The yield of each step will be added. The results obtained for different solvents and raw material are shown in the following tables:

Table 5.5.1 Dynamic maceration extraction results for the original Thymus with 96% ethanol

96% Ethanol	**Dry Thymus (g)**		**9,19**
	First step	Second step	Third step
Empty flask	115,925	130,88	138,26
Flask + extract	116,61	131,38	138,39
Extract	0,69	0,50	0,13
Residue	8,51	8,01	7,88
Yield (%)	7,45	5,44	1,41
Sumatory (%)		***14,31***	

Table 5.5.2 Dynamic maceration extraction results for the original Thymus with 50:50 ethanol-water

50% Ethanol 50% water	**Dry Thymus (g)**		**9,075**
	First step	Second step	Third step
Empty flask	111,06	112,67	96
Flask + extract	112,59	113,3	96,29
Extract	1,53	0,63	0,29
Residue	7,55	6,92	6,63
Yield (%)	16,86	6,94	3,20
Sumatory (%)		***27,00***	

Table 5.5.3 Dynamic maceration extraction results for the original Thymus with water

Water	Dry Thymus (g)		9,62
	First step	Second step	Third step
Empty flask	111,1	133,31	122,55
Flask + extract	113,32	133,85	122,79
Extract	2,22	0,54	0,24
Residue	7,40	6,86	6,62
Yield (%)	23,08	5,61	2,49
Sumatory (%)		***31,19***	

Table 5.5.4 Dynamic maceration extraction results for the residual Thymus with 96% ethanol

96% Ethanol Residue	Dry Thymus (g)		10,15
	First step	Second step	Third step
Empty flask	120,95	121,85	120,96
Flask + extract	121,81	122,13	121,12
Extract	0,86	0,28	0,16
Residue	9,29	9,01	8,85
Yield (%)	8,47	2,76	1,58
Sumatory (%)		***12,81***	

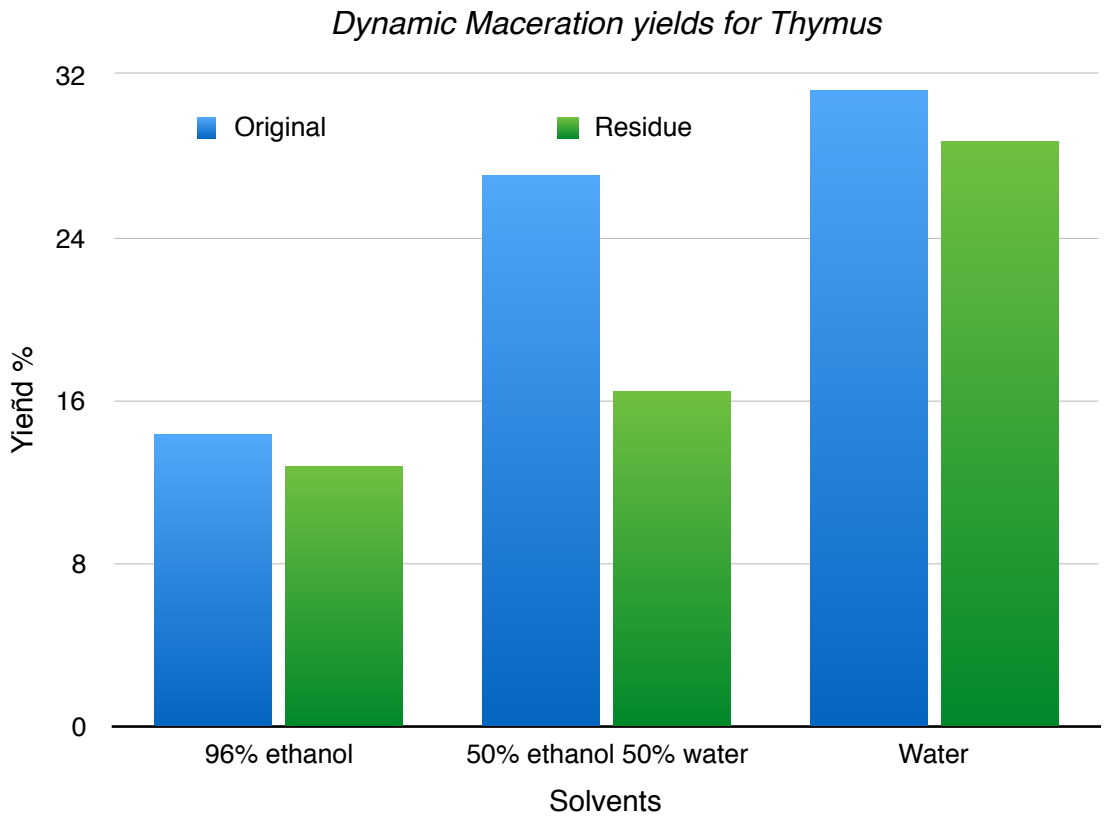
Table 5.5.5 Dynamic maceration extraction results for the residual Thymus with 50:50 ethanol-water

50% ethanol 50% water residue	Dry Thymus (g)		9,93
	First step	Second step	Third step
Empty flask	96,03	121,81	130,95
Flask + extract	98,27	122,12	131,02
Extract	2,24	0,31	0,07
Residue	7,69	7,38	7,31

50% ethanol 50% water residue	Dry Thymus (g)		9,93
Yield (%)	22,56	3,12	0,70
Sumatory (%)		***26,38***	

Table 5.5.6 Dynamic maceration extraction results for the residual Thymus with water

Water residue	Dry Thymus (g)		10,78
	First step	Second step	Third step
Empty flask	121,87	113,55	130,48
Flask + extract	124,04	114,22	130,73
Extract	2,17	0,67	0,25
Residue	8,61	7,94	7,69
Yield (%)	20,13	6,22	2,32
Sumatory (%)		***28,66***	



As in the Soxhlet extraction, the highest extraction yield came from the most polar solvent, in this case the water; the mixture of compound with different polarities can extract a larger range of molecules.

In the case of the residue, we can also conclude the same, and remark that happens the same that in Soxhlet, it means that the yields difference between the original and the residue lies in experimental deviation errors, and like in the soxhlet method, the difference can also came from the properties that the essential oil retain (it means that only stay in the pure raw).

At the view of the results, it is easy to observe that the yield between the first and the third step are quite big, so as conclusion the experiment could be done only with two steps without a big error.

5.6 Steam distillation + refraction index

In this distillation, the only measurement done has been the volume of the essential oil obtained and the measure of the refraction index:

Table 5.6.1 Yield of steam distillation and refraction index

Essential oil	Volume (ml)	Plant weight (g)	Yield (%)	Refraction index
1st measurement	0,63	52,94	1,19	1,4992
2nd measurement	0,71	50,42	1,41	1,4994
3rd measurement	0,65	49,2	1,32	1,4996
Average	0,66	50,85	1,31	1,4994

The yield obtained is 1,31 ± **0,11%.**

The error committed is negligible, being the average of the refraction 1,4994 ± **0,0002** (refraction index have not index).

The refraction index has been compared with the Sigma-Aldrich Thymus average (1,489-1,51) . Then, looking at the results, it is possible to conclude that the essential oil extraction is correct. The yield also must be between 1-2%, so are within the parameters.

The steam distillation was also done with the residues, without obtaining essential oil, which means that the steam distillation in the pilot plant was successful.

5.7 Spectrophotometer results

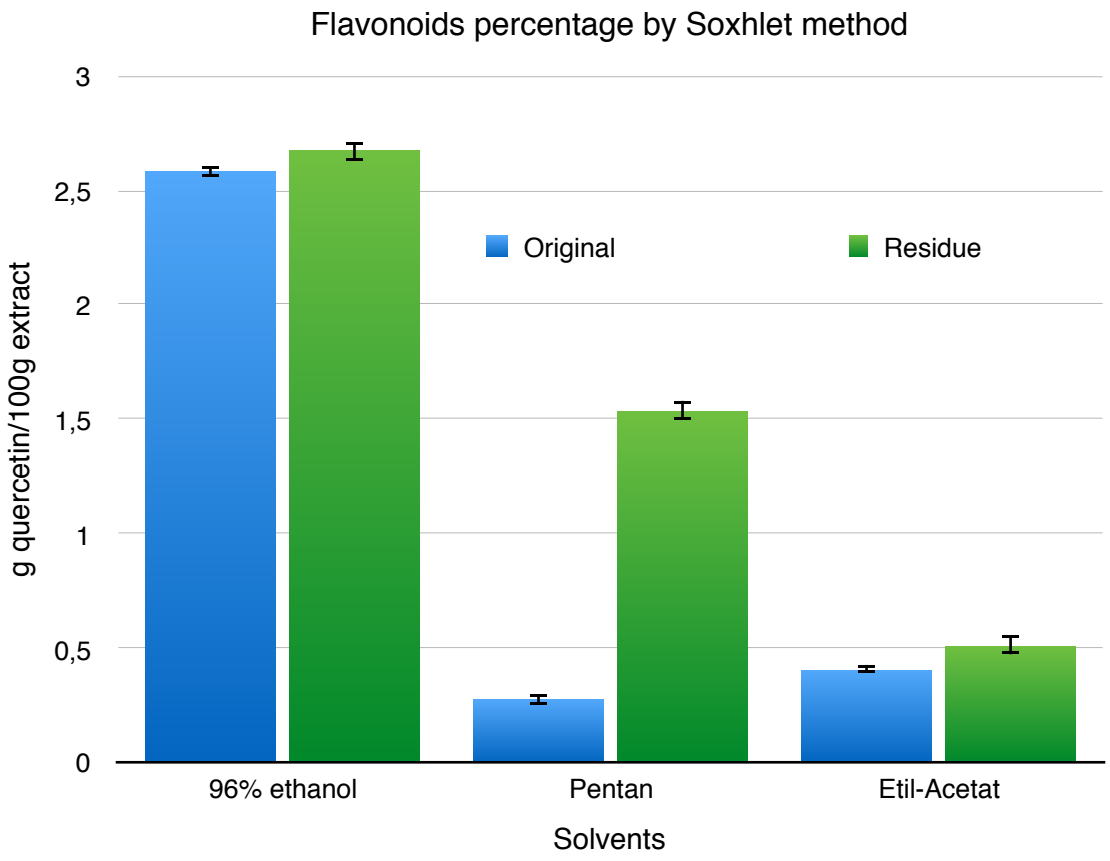
A large number of analysis of antioxidants, flavonoids, polyphenols and tannins has been done, each one with his own preparation method. The results obtained are the next:

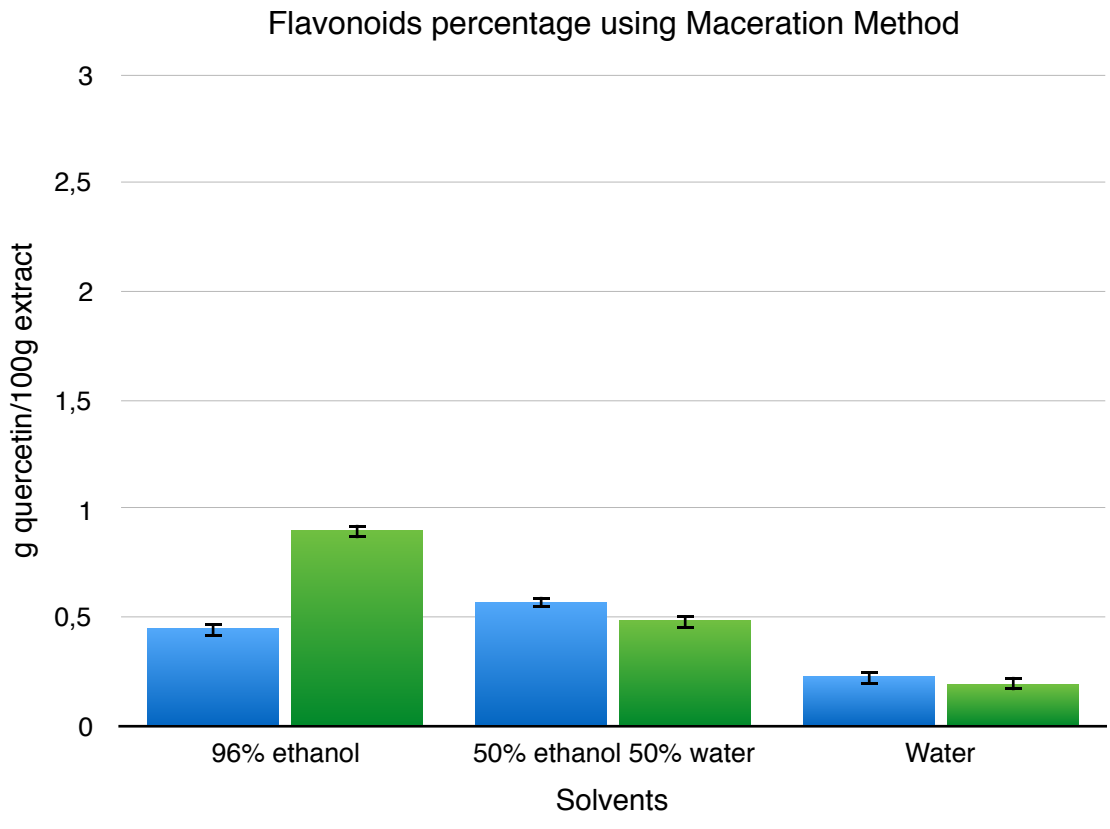
5.7.1 Flavonoids

5..7.1.1 Flavonoid content

	Absorbance	% flavonoids	Standard deviation
Soxhlet 96% Or	0,736	2,583	0,031
Soxhlet 96% Res	0,432	2,672	0,080
Soxhlet pentan or	0,019	0,278	0,016
Soxhlet pentan res	0,095	1,535	0,005
Soxhlet acet or	0,050	0,410	0,018
Soxhlet acate res	0,430	0,513	0,008
Maceration et orig	0,040	0,442	0,010
Maceration et res	0,073	0,900	0,008
Maceration 50 50 orig	0,081	0,569	0,017
Maceration 50 50 res	0,047	0,482	0,021
Macer water	0,018	0,224	0,010
Macer water residue	0,019	0,190	0,009

The % of flavonoids is a unit obtained from the division between g of querecitin/100g of extract. Quercitin is one reference used for the calibration lines.


Flavonoids percentage by Soxhlet method
3
2,5
2
1,5
1
0,5
0
g quercetin/100g extract
Original
Residue
96% ethanol
Pentan
Etil-Acetat
Solvents


Flavonoids percentage using Maceration Method
3
2,5
2
1,5
1
0,5
0
g quercetin/100g extract
96% ethanol
50% ethanol 50% water
Water
Solvents

It is possible to conclude under this results that the percentage of flavonoids in the Thymus vulgaris is really low, being the best percentage with ethanol with the Soxhlet method.

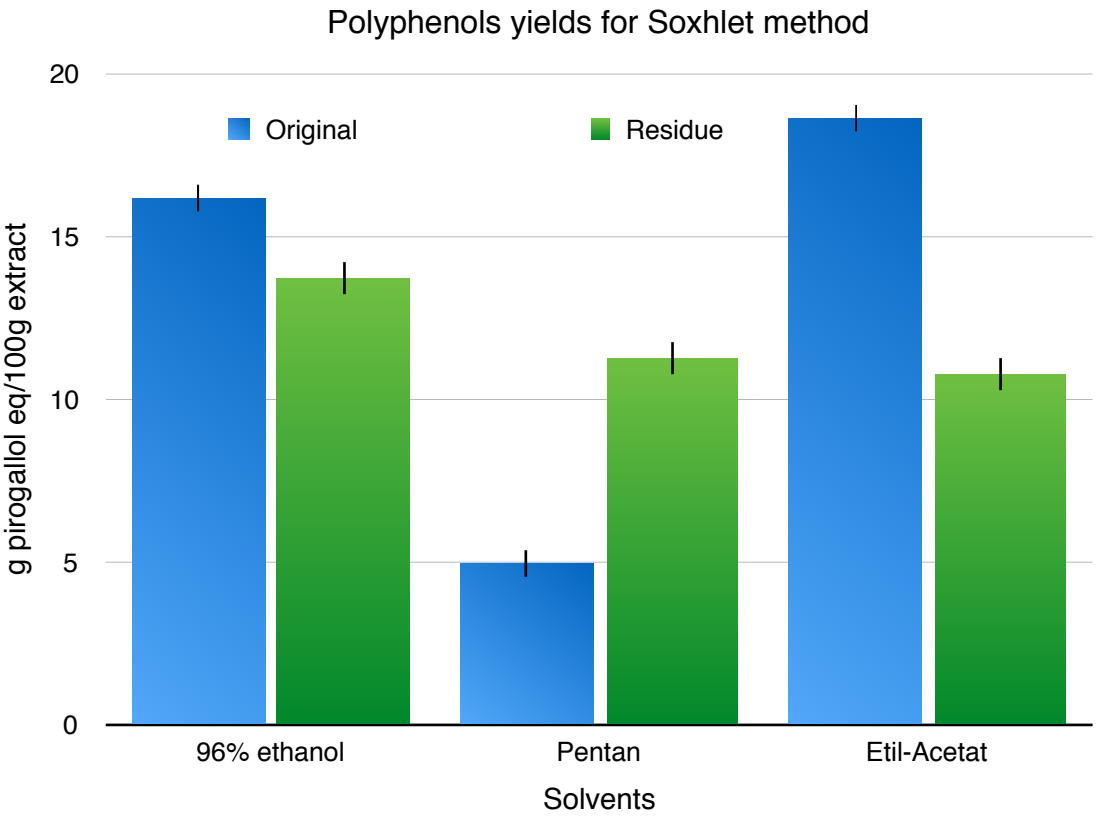
The difference between original raw and the residue is not important, while the standard deviation is quite small en each experiment, thereupon it is possible to conclude that there are not difference between pure and residue.

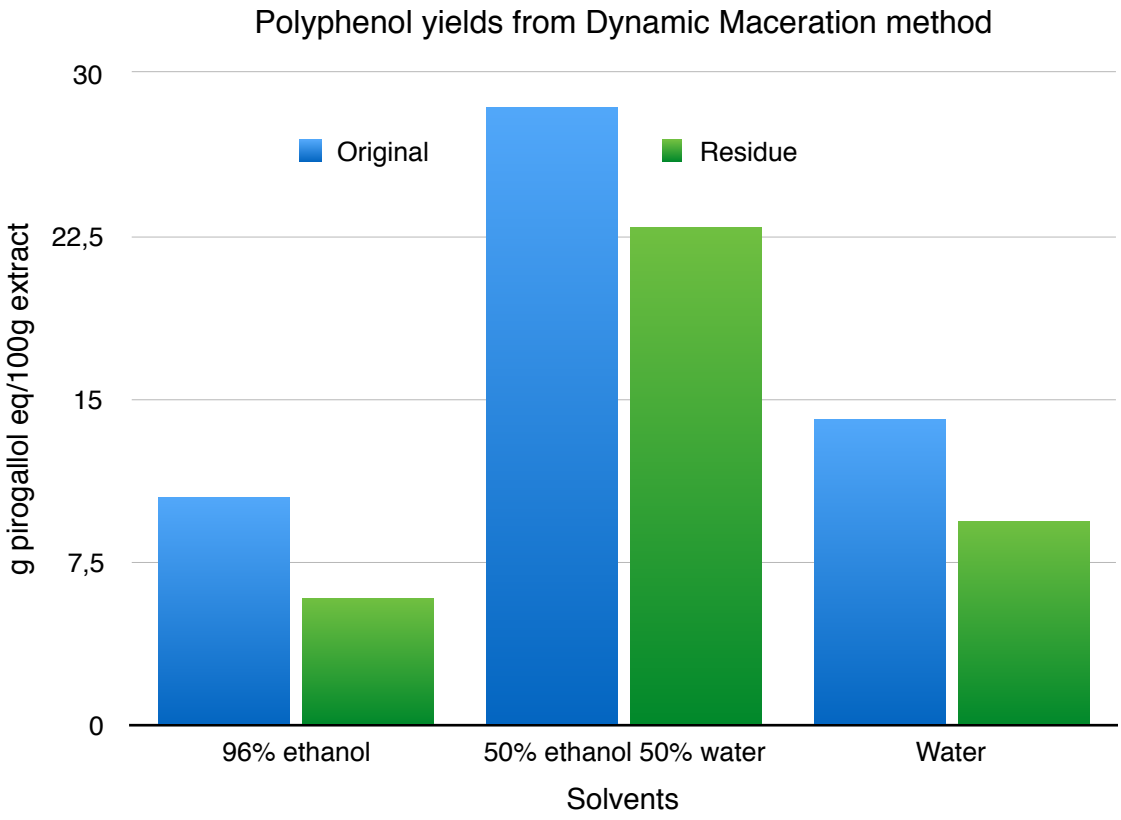
5.7.2 Polyphenols

The % of polyphenols is a unit obtained from the division between g of pirogallol/100g of extract. Pirogallol is one reference used for the calibration lines.

5.7.2.1. Polyphenol content

	Absorbance	% polyphenols	Standard deviation
Soxhlet 96% Or	2,052	16,189	0,015
Soxhlet 96% Res	0,986	13,720	0,009
Soxhlet pentan or	0,150	4,936	0,012
Soxhlet pentan res	0,309	11,273	0,008
Soxhlet acet or	0,510	18,598	0,009
Soxhlet acate res	0,295	10,739	0,005
Maceration et orig	0,288	10,508	0,008
Maceration et res	0,195	5,835	0,008
Maceration 50 50 orig	0,779	28,389	0,014
Maceration 50 50 res	0,628	22,898	0,004
Macer water	0,385	14,043	0,010
Macer water residue	0,257	9,378	0,040


Polyphenols yields for Soxhlet method
20
15
10
5
0
g pirogallol eq/100g extract
Original
Residue
96% ethanol
Pentan
Etil-Acetat
Solvents


Polyphenol yields from Dynamic Maceration method
30
22,5
15
7,5
0
g pirogallol eq/100g extract
Original
Residue
96% ethanol
50% ethanol 50% water
Water
Solvents

In the graphics above it is possible to see that in the Soxhlet method the yield is similar between the ethanol and the ethyl-acetate. However, in the dynamic maceration, the yield of the mixture water-ethanol 50:50 is much more higher that the rest of experiments, reaching almost the 30% of polyphenols content. This is due to the polarity of the mixture is more suitable for *Thymus* components than are mainly polar and semi-polar, than the pure solvents by their own.

Respecting to the residue, the yield is lower than the original, around 2-3% in all the experiments, except the pentane. The reason of that can be a thermal degradation during the steam distillation.

The difference is quite similar between original raw and the residue.

5.7.3 Tannins

5.7.3.1. Non-tannin polyphenol content

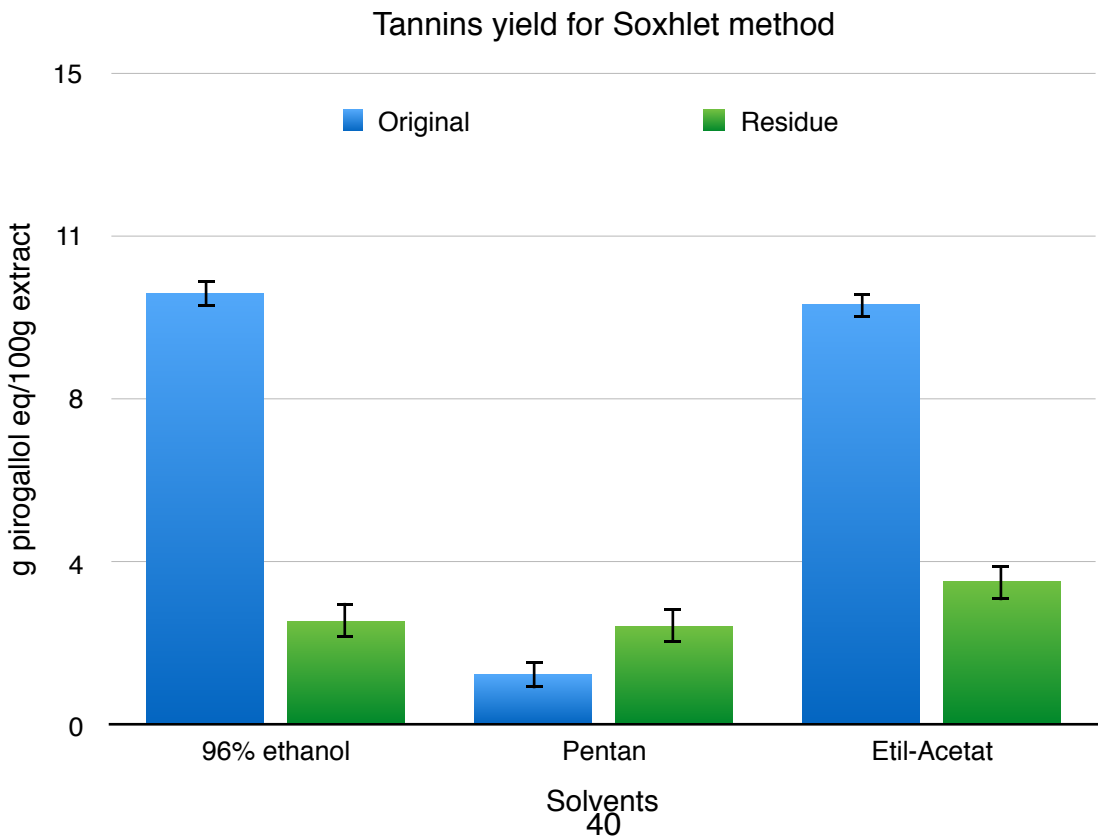
	Absorbance	% tannins	Standard deviation
Soxhlet 96% Or	0,844	6,655	0,015
Soxhlet 96% Res	0,813	11,313	0,009
Soxhlet pentan or	0,116	3,798	0,012
Soxhlet pentan res	0,108	1,982	0,004
Soxhlet acet or	0,485	8,933	0,009
Soxhlet acate res	0,280	7,448	0,009
Maceration et orig	0,170	4,186	0,008
Maceration et res	0,157	3,954	0,008
Maceration 50 50 orig	0,660	10,407	0,014
Maceration 50 50 res	0,504	11,691	0,004
Macer water	0,315	8,631	0,010
Macer water residue	0,243	5,570	0,004

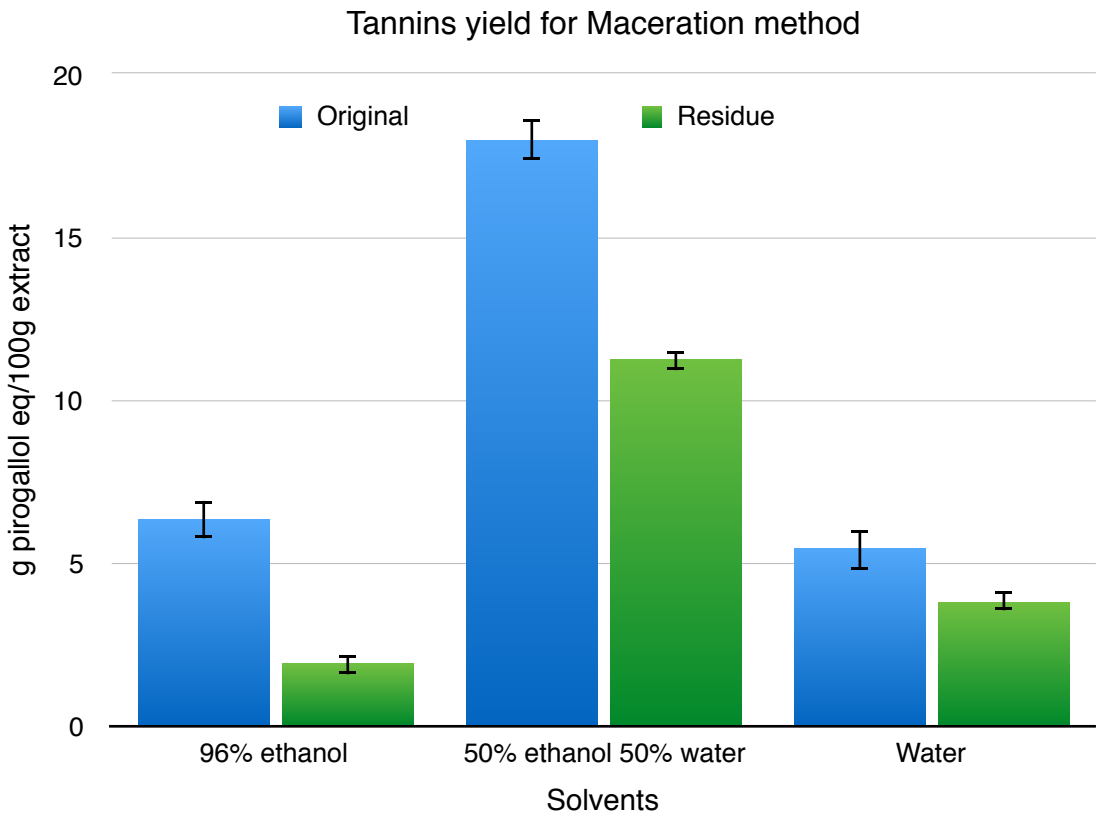
The % of tannins is a unit obtained from the division between g of pirogallol/100g of extract. Pirogallol is one reference used for the calibration lines.

The tannins measured are not real, the measurement done is the part of the polyphenols that not contains tannins, so we have to subtract this value to the polyphenols, obtaining the tannins. Te results will be shown in the next table:

5.7.3.2. Tannins content

	% Real Tannins	Standard deviation
Soxhlet 96% Or	9,534	0,015
Soxhlet 96% Res	2,407	0,009
Soxhlet pentan or	1,138	0,012
Soxhlet pentan res	9,291	0,004
Soxhlet acet or	9,665	0,009
Soxhlet acate res	3,291	0,009
Maceration et orig	6,322	0,008
Maceration et res	1,881	0,008
Maceration 50 50 orig	17,982	0,014
Maceration 50 50 res	11,207	0,004
Macer water	5,412	0,010
Macer water residue	3,808	0,004





It is possible to conclude that all the original raw have a better percentage of tannins that the residue (except pentane, but the % in this solvent is not good enough). And the difference between original and residue could be explained for the creation of free radicals during the steam distillation, which maintain the polyphenol appearance, but there are not tannins for more.

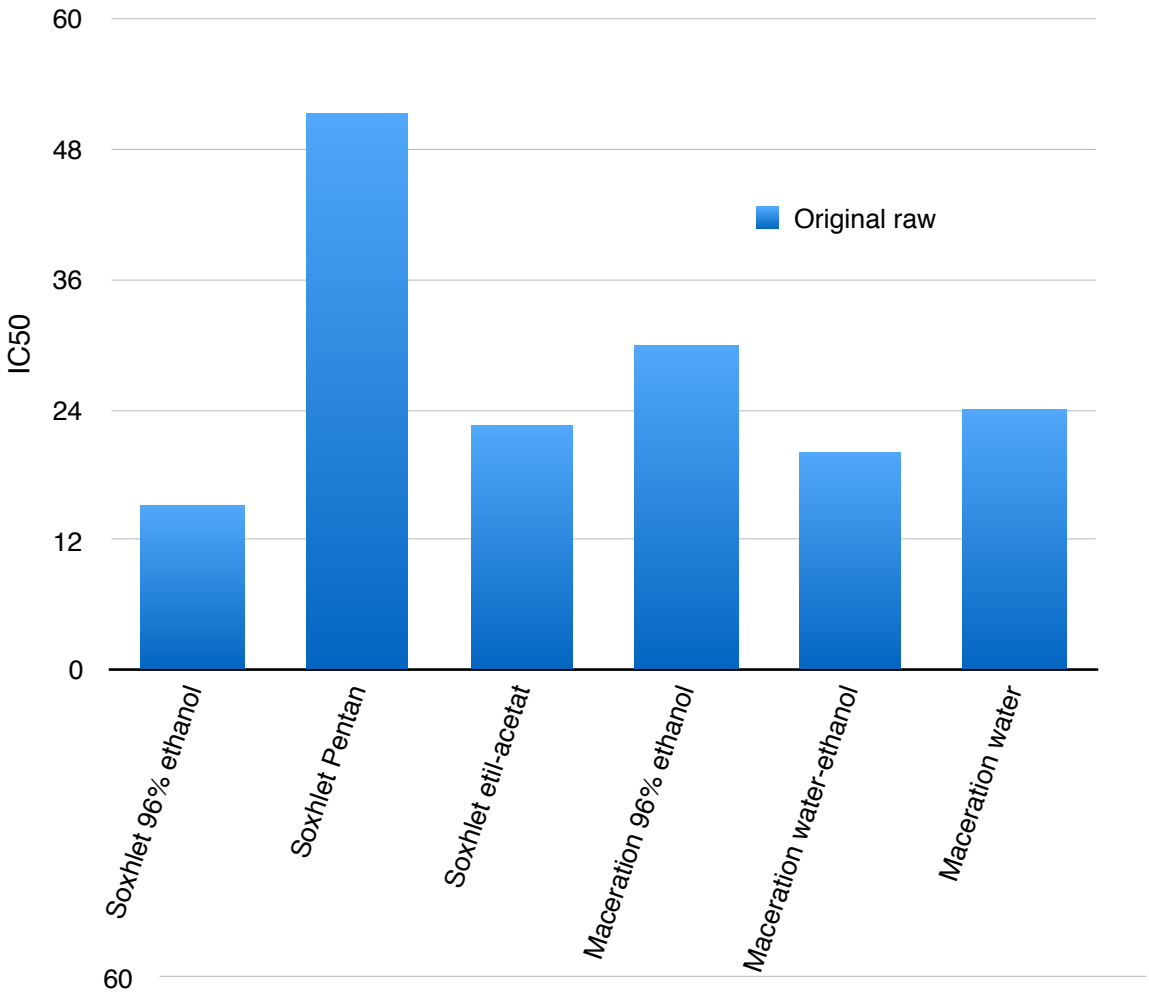
The highest percentage of tannins belong to the 96% ethanol in the soxhlet method, and to the mixture 50:50 ethanol-water mixture in the dynamic maceration, which have the best yield.

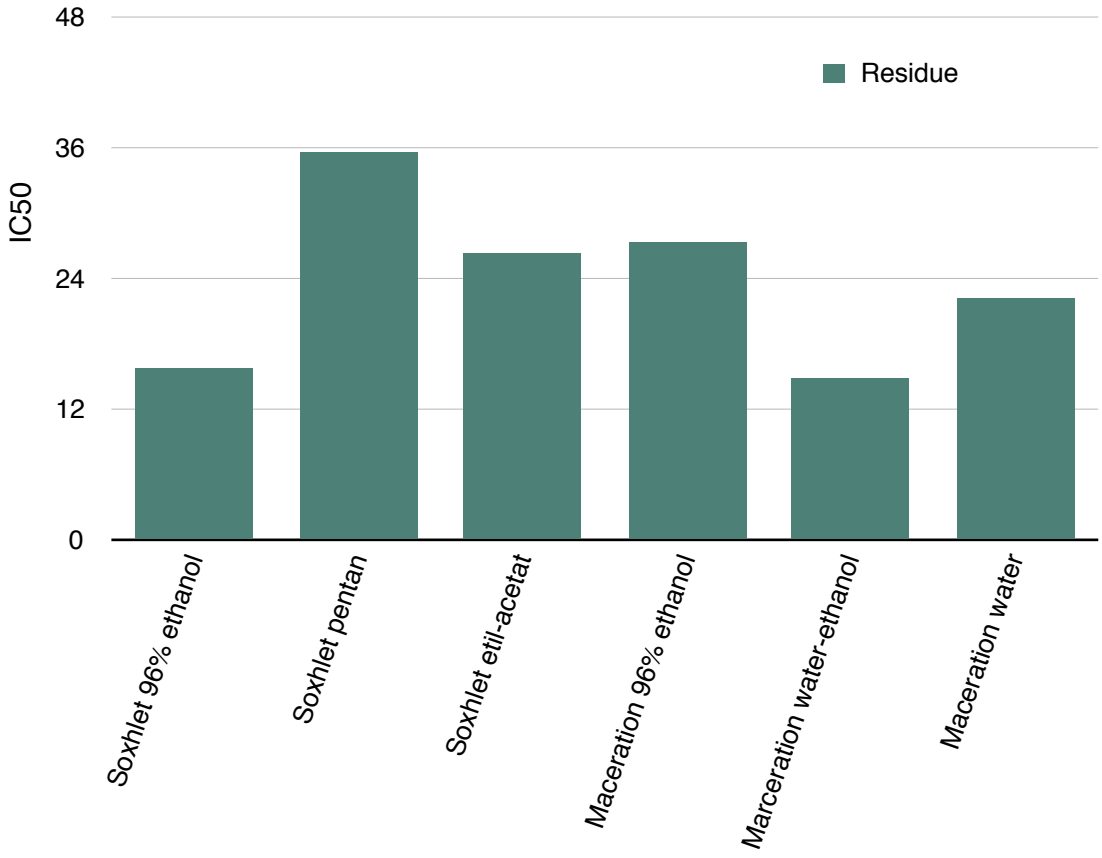
That it's according to the expectation, because tannins are polar compounds.

5.7.4 Antioxidants

Two measurements has been done of each extract, calculating in each case the average and the standard deviation.

ANTIOXIDANTS		
	IC50	***Standard deviation***
Soxhlet 96% ethanol original	15,23	0,78
Soxhlet 96% ethanol residue	15,83	1,23
Soxhlet Pentan original	51,35	1,6
Soxhlet Pentan residue	35,58	2,29
Soxhlet etil-acetat original	22,62	3,21
Soxhlet etil-acetat residue	26,27	1,98
Maceration 96% ethanol original	29,88	4,58
Maceration 96% ethanol residue	27,2	0,8
Maceration water-ethanol original	20,07	2,75
Maceration water-ethanol residue	14,71	1,41
Maceration water original	24,01	0,12
Maceration water residue	22,06	0,38


60
48
36
24
12
0
IC50
Original raw
Soxhlet 96% ethanol
Soxhlet Pentan
Soxhlet etil-acetat
Maceration 96% ethanol
Maceration water-ethanol
Maceration water


60
48
36
24
12
0
IC50
Residue
Soxhlet 96% ethanol
Soxhlet pentan
Soxhlet etil-acetat
Maceration 96% ethanol
Marceration water-ethanol
Maceration water

Knowing that the IC50 is the concentration of the solution at 50% (where the amount of the antioxidants is the half in the start), it is obvious that the inhibition power of the pentane is higher than the rest, and that the best methods (with the smallest IC50), are the 96% ethanol in the soxhlet and the 50:50 ethanol-water in the maceration.

Than can be explained with the polarities, because the *n*-pentane is apolar, while with the rest the polarity is becoming higher (ethanol and water > ethyl acetate).

6.Conclusions

A large study about organic components extraction from Thymus Vulgaris has been carried out. The study has taken into account different methods of extraction, solvents, and the spectrophotometer analysis.

Essential oil was extracted using the steam distillation method and yielded (1,34 ± ***0,22).*** Then the essential oil was characterized with refractometry and fell within the required limits (1,4994 ± ***0,0002).***

Soxhlet extraction has been performed with solvents of different polarities. The best yields were obtained with the 96% ethanol (***27,3 ± 3,3%).*** This is due to the polarity of the solvent is more suitable for *Thymus'* components that are mainly polar. In the same way, maceration extraction was performed with different solvents. The best yield obtained was the one with water as solvent (**31,19**) for the same reason, the polarity of the water is higher than the rest.

In the bioactivity analysis (flavonoids, polyphenols and tannins), the results shows that the highest concentrations are obtained with ethanol 96% in the soxhlet, and in the maceration, the 50:50 ethanol-water mixture. As exception, the highest concentration (by far) in the antioxidants analysis belongs to the pentane solvent, being the concentration of the rest of the compounds the lowest one.

In terms antioxidants, the IC50 shows an inhibition power concentration, so as lower were the values, the antioxidant capacity becomes better. The best antioxidant capacity with the 96% Ethanol Soxhlet method (15,23 ± ***0,78)***.

After a steam distillation in a pilot plant with a big amount of raw (around 5 kilograms), a residue obtained has been analyzed to find out the difference with the original raw in the distillation is around 1-2%, which can be blamed to the common experimental errors. Besides, the bioactive analysis from the residue look like the original, being a bit lower than them, and being even bigger in some isolated case. The highest percentage of flavonoids is (2,672 ± ***0,08)*** obtained by the 96% soxhlet residue. The highest percentage of polyphenols is (28,389 ± ***0,014)*** obtained by the 50:50 water-ethanol maceration of pure raw. The highest percentage of tannins is (17,982 ± ***0,14)*** that has been obtained by the 50:50 maceration of pure raw**.** The best IC50 (it means the lowest) of antioxidants is (15,23 ± ***0,78),*** which belongs to the 96% ethanol soxhlet method.

Finally, we can conclude that the best Soxhlet solvent will be the 96% ethanol, and in the dynamic maceration method, the best will be the mixture 50:50 ethanol-water.

7.Literature

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8. Appendix

Antioxidants

Pentan Soxhlet			
Concentration (µg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
41,85	0,34	0,28	11,3
45,70	0,42	0,35	Liquid (ml)
49,49	0,47	0,43	20
53,22	0,51	0,56	C0 (mg/ml)
56,90	0,57	0,65	0,565
60,54	0,6	0,71	Average
IC50	52,48	50,22	51,35
Standard deviation	1,6		

Pentan residue Soxhlet			
Concentration (µg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
41,85	0,19	0,13	
45,70	0,28	0,22	Liquid (ml)
49,49	0,38	0,35	
53,22	0,5	0,45	C0 (mg/ml)
56,90	0,58	0,53	
			Average
IC50	33,76	37	35,38
Standard deviation		2,29	

96% ethanol Soxhlet			
Concentration (µg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)

96% ethanol Soxhlet			
4,48	0,18	0,17	11,3
8,9	0,34	0,31	Liquid (ml)
13,24	0,45	0,41	20
17,52	0,58	0,54	C0 (mg/ml)
21,73	0,61	0,64	0,565
			Average
IC50	14,68	15,78	15,23
Standard deviation	0,78		

96% ethanol residue Soxhlet			
Concentration (µg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
4,37	0,07	0,07	11
8,66	0,3	0,27	Liquid (ml)
12,89	0,44	0,39	20
17,05	0,58	0,49	C0 (mg/ml)
21,15	0,73	0,6	0,55
			Average
IC50	14,86	16,79	15,83
Standard deviation		1,36	

Etil-Acetat Soxhlet			
Concentration (µg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
13,95	0,3	0,35	11,9
18,45	0,37	0,46	Liquid (ml)
22,89	0,45	0,55	20
27,25	0,54	0,6	C0 (mg/ml)
31,55	0,63	0,69	0,595

Etil-Acetat Soxhlet			
			Average
IC50	24,89	20,35	22,62
Standard deviation	3,21		

Etil-Acetat Residue Soxhlet			
Concentration (µg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
22,67	0,42	0,46	9,9
26,25	0,46	0,53	Liquid (ml)
29,77	0,53	0,6	20
33,25	0,61	0,65	C0 (mg/ml)
36,67	0,67	0,72	0,495
			Average
IC50	27,67	24,87	26,27
Standard deviation	1,98		

Maceration 96% ethanol			
Concentration (µg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
22,9	0,42	0,46	10
26,52	0,51	0,55	Liquid (ml)
30,08	0,55	0,6	20
33,58	0,66	0,61	C0 (mg/ml)
37,04	0,72	0,63	0,5
			Average
IC50	26,64	33,12	29,88
Standard deviation		4,58	

Maceration 96% ethanol residue			
Concentration (μg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
25,42	0,47	0,53	11,1
29,43	0,53	0,54	Liquid (ml)
33,38	0,59	0,61	20
37,27	0,68	0,71	C0 (mg/ml)
41,11	0,75	0,72	0,555
			Average
IC50	26,64	27,76	27,2
Standard deviation	0,79		

Maceration ethanol-water 50:50 original			
Concentration (μg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
4,68	0,18	0,23	11,8
9,29	0,29	0,36	Liquid (ml)
13,82	0,41	0,47	20
16,07	0,46	0,5	C0 (mg/ml)
18,3	0,53	0,57	0,59
			Average
IC50	18,11	22,01	20,07
Standard deviation	2,75		

Maceration ethanol-water 50:50 residue			
Concentration (μg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
5,04	0,18	0,13	12,7
10	0,36	0,32	Liquid (ml)
14,88	0,47	0,59	20
17,3	0,52	0,67	C0 (mg/ml)
19,69	0,64	0,74	0,635
			Average
IC50	15,71	13,71	14,71
Standard deviation	1,41		

Maceration water original			
Concentration (μg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
14,3	0,34	0,36	12,2
18,91	0,41	0,45	Liquid (ml)
23,46	0,49	0,53	20
27,94	0,59	0,58	C0 (mg/ml)
32,35	0,66	0,69	0,61
			Average
IC50	22,14	21,97	22,06
Standard deviation	0,12		

Maceration water residue			
Concentration (μg/ml)	Inhibition 1	Inhibition 2	
0	0	0	Solid (mg)
18,91	0,34	0,36	12,2
23,46	45	0,46	Liquid (ml)
27,94	0,5	0,54	20
31,25	0,63	0,66	C0 (mg/ml)

Maceration water residue			
34,52	0,69	0,73	0,61
			Average
IC50	26,74	24,28	24,01
Standard deviation	0,38		

Siceving

Weight	sieve	tara	tara + extracto	extract	%	Reverse
60,03	0	352,48	352,48	0,00	0,00	0,00
	0,1	241,8	241,95	0,15	0,25	0,00
	0,315	288,67	290,35	1,68	2,80	0,25
	0,5	306,22	315,18	8,96	14,93	3,05
	0,63	381,47	403,22	21,75	36,23	17,97
	0,8	399	417,48	18,48	30,78	54,21
	1	402,35	411,36	9,01	15,01	84,99
					100,00	100
Peso	sieve	tara	tara + extracto	maradek	%	Reverse
59,78	0	362,48	362,48	0,00	0,00	0,03
	0,1	241,8	241,95	0,15	0,25	0,03
	0,315	288,7	288,85	0,15	0,25	0,28
	0,4	358,9	360	1,10	1,83	0,53
	0,5	306,21	313,7	7,49	12,48	2,37
	0,63	381,5	395,62	14,12	23,52	14,84
	0,8	398,9	435,9	37,00	61,64	38,36
					100	100
Weight	sieve	tara	tara + extracto	extract	%	Reverse
62,08	0	352,48	352,48	0,00	0,00	0,00
	0,1	241,8	241,97	0,17	0,27	0,00
	0,315	288,67	290,55	1,88	3,03	0,27
	0,5	306,22	316,66	10,44	16,82	3,30
	0,63	381,47	404,41	22,94	36,95	20,12
	0,8	399	416,99	17,99	28,98	57,07
	1	402,35	411,01	8,66	13,95	86,05
					100,00	100

Obtention of density

Pure raw

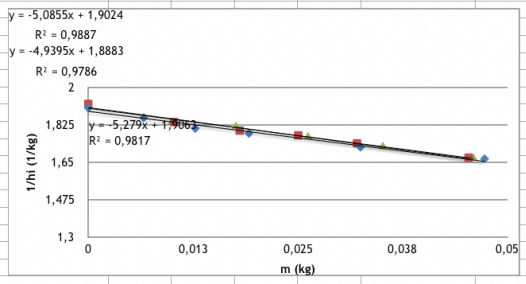
i	hi (m)	mi (kg)	1/hi (1/kg)
1	0,525	0	1,904761904
2	0,538	0,00661	1,858736059
3	0,553	0,01276	1,808318264
4	0,560	0,01916	1,786
5	0,581	0,03248	1,721170395
6	0,6	0,04728	1,666666666

i	hi (m)	mi (kg)	1/hi (1/kg)
1	0,52	0	1,923076923
2	0,544	0,01023	1,838235294
3	0,556	0,01806	1,798561151
4	0,563	0,02505	1,776198934
5	0,575	0,03206	1,739130434
6	0,598	0,04537	1,672240802

i	hi (m)	mi (kg)	1/hi (1/kg)
1	0,523	0	1,912045889
2	0,545	0,0099	1,834862385
3	0,55	0,01761	1,818181818
4	0,565	0,02622	1,769911504
5	,58	0,03515	1,724137931
6	0,599	0,04595	1,669449081

	gradient	T(laboratory) (˚C)	ρ(water)	g (m/s²)	p0 (Pa)	VAB (m3)	ρ(plant) (kg/m3)
1	-4,9395	25	997	9,81	101831	0,000015	1296,31309514171
2	-5,279		997	9,81	101831	0,000015	1212,94535583491
3	-5,0855		997	9,81	101831	0,000015	1259,09714550241

Average density: 1.256,12



Thymus residue

i	hi (m)	mi (kg)		1/hi (1/kg)
1	0,523	0,000	0	1,912
2	0,539	0,011	0,0108	1,855
3	0,557	0,021	0,0210	1,795
4	0,568	0,031	0,0312	1,761
5	0,599	0,045	0,0446	1,669

i	hi (m)			1/hi (1/kg)
1	0,521	0,000	0,0000	1,919
2	0,533	0,009	0,0087	1,876
3	0,550	0,018	0,0184	1,818
4	0,570	0,026	0,0262	1,754
5	0,582	0,033	0,0325	1,718
6	0,601	0,047	0,0465	1,664

i	hi (m)			1/hi (1/kg)
		0,000		
1	0,524	0,000	0,0000	1,908
2	0,531	0,007	0,0072	1,883
3	0,548	0,016	0,0162	1,825
4	0,565	0,025	0,0247	1,770
5	0,583	0,032	0,0324	1,715
6	0,601	0,045	0,0454	1,664

	gradient	T(laboratory) (˚C)	ρ(water)	g (m/s²)	p0 (Pa)	VAB (m3)	ρ(plant) (kg/m3)
1	-5,6965	25	997	9,81	101831	0,000015	1124,04784226323
2	-5,3051		997	9,81	101831	0,000015	1206,97791435647
3	-5,7658		997	9,81	101831	0,000015	1110,5377455778
							1147,18783406583