Dissertation for the PhD Degree

Effects of Supercritical Carbon Dioxide and Sub-critical Propane Extraction of Thyme and Cardamom on Chemical Composition, Antioxidant Capacity and Antimicrobial Properties

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CONTENTS

1. INTRODUCTION

There have been great efforts to prepare safe and potent extract from natural products that have nutritional, industrial and therapeutical importance. Cardamom and thyme are aromatic herbs that are used extensively to add a distinctive aroma and flavor to food beside their gastro-protective and anti-microbial activity.

With the progress in technology and increasing level of environmental pollution, there is an increasing demand to produce safe foods or food ingredients with high antioxidant capacity and high content of bioactive compounds. World wide and particularly in the Middle East countries incorporation of thyme and cardamom products in preparing of different meals has become one of the well known traditions and habits for the nations of the area.

Since fresh products of the two plants are not available around the year; production of their extracts is of special interest.

Our developed world has a strong demand for creation healthy and less polluted environment using clean technologies and producing solvent residue free, healthy foods. The food products must posses natural colour, taste and shelf-live extensive properties and should contain biological active, health preventive compounds (e.g antioxidants, vitamins) as well. Supercritical fluid extraction is one of the desirable technologies, which uses mainly carbon dioxide alone or with modifiers for the extraction of essential oils, pigments, and natural waxes from natural sources, mainly from herbs, spices and medicinal plants. According to the physico-chemical properties of supercritical and subcritical fluids, the extraction is carried out at a moderate temperature (mainly between 25-60 ˚C) , therefore thermo-labile compounds can be obtained without any decomposition. The extract is absolutely solvent residual free as the CO_2 is in gaseous state at room temperature. Numerous, mainly apolar , compounds can be extracted and |or fractionated as the solvating power of supercritical and subcritical fluids changes within a wide range with the changes of the pressure (in case of sub-critical fluid) and pressure and temperature (in case of supercritical ones) during extraction. Products of biological origin are often thermally labile, lipophilic, non volatile, and required to be kept around room temperature. Carbon dioxide (CO_2) has a critical temperature of 31˚C which makes it a particulary proper medium for the extraction of biological materials.

Objectives of the work:

The main objective of this work was to optimize the conditions of supercritical carbon dioxide and sub-critical propane to achieve maximum recovery of active substances in oleoresins from wild thyme and cardamom seeds. The tasks undertaken in research work are summarized as follows:

1. Optimization of supercritical CO_2 and sub-critical propane for the best extract of oleoresin and fat-soluble bioactive and volatile compounds from thyme and cardamom.
2. Use of ethanol as a modifier to increase recovery of oleoresins and to improve solubility of bioactive compounds.
3. Application of recent analytical method to determine the active substances with special focus on the natural pigments, antioxidants and compounds in the raw material and extracts.
4. Studying the changes in the antioxidant content and activity of thyme and cardamom extracts as a function of SFE conditions.
5. Investigation of anti-microbial activity of thyme and cardamom extracts produced under different conditions.

2. LITERATURE REVIEW

2.1. Supercritical fluid extraction

2.1.1. *Introduction to supercritical fluids*

The discovery of supercritical state is attributed to Baron Cagniard de le Tour in 1822 , who heated alcohol in a sealed gun barrel and listened to the musket ball rolling about.This acoustic effect caused by the fluctution when the fluid passed through the critical point. He also observed that the boundary between a gas and a liquid disappeared for certain substances when the temperature was increased in a sealed gas container. The ability of a supercritical fluid to dissolve low-vapor-pressure solid materials was first reported by Hanny and Hogarth in 1879. They observed that increasing the pressure caused several inorganic salts (e.g. cobalt chloride , potassium iodide, and potassium bromide) to dissolve in ethanol at a temperature above the critical temperature of ethanol (Tc=243˚C ; Pc=63 bar). Until 1960-ies the tests on supercritical materials (fluids) were confined to their solubility and phase behavior. Since then researchers were interested in using supercritical fluids for material separation in industry of coffee, hop, etc. A major development was the installation by Kraft General Foods of a decaffeination plant to their Maxwell House Coffee Division in 1987, which uses an extraction cell with a height of 25m. Supercritical fluids now encompass a multidisciplinary field that includes engineers, chemists, food scientists, material scientists, and workers in biotechnology, agriculture and environmental control *(Pellerin, 1991; McHugh and Krukonis, 1994; Dean, 1998 ; Tomasko et al., 1999; Mukhopadhyay ; 2000 Poliakoff and King,2001.)*

When a gas is compressed to a sufficiently high pressure, it becomes liquid. If, on the other hand, the gas is heated beyond a specific temperature, no amount of compression of the hot gas will cause it to become a liquid. This temperature is called the critical temperature (Tc) and the corresponding vapor pressure is called the critical pressure (Pc). These values of temperature and pressure define a critical point which is unique to a given substance. The state of substance is called supercritical fluid (SCF) when both the temperature and pressure exceed the critical point values, but if one parameter (usually the pressure) is over the critical value, it is a sub-critical fluid (*Simándi and Sawinsky, 1996 ; Mukhopadhyay, 2000 ; Gamse, 2000 ; Oszagyán et al., 2000 ; Simándi et al., 2000).*

Figure 1 shows the phase diagram of solid, liquid, and gas phases with their areas. Point C indicates the critical point. As the critical point of a substance is approached, its isothermal compressibility tends to infinity, thus its molar volume or density changes dramatically.

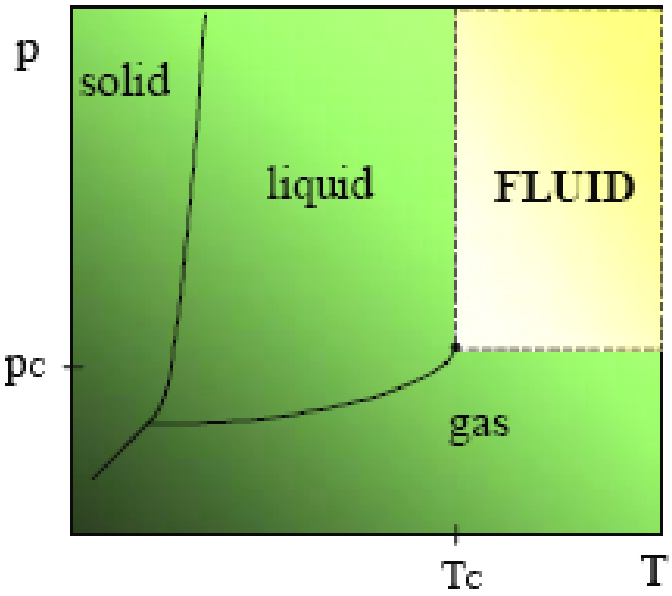


Figure 1 P-T phase diagram

In the critical region a substance that is a gas at normal conditions exhibits a liquid-like density and a much increased solvent capacity that is pressure-dependent *(McHugh and Krukonis,1994).* A comparison of typical values for density, viscosity and diffusivity of gases, liquids, and supercritical fluids (SCF) is presented in Table 1.

Table 1: Comparison of physical and transport properties of gases, liquids and SCFs

Property	**Gas**	**SCF**	**Liquid**
Density (kg /m³)	**1**	**200-700**	**1000**
Viscosity (Pa .s)	**10^{-5}**	**10^{-4}**	**10^{-3}**
Diffusivity (cm² /s)	**10^{-1}**	**10^{-3}-10^{-4}**	**10^{-5}**

In the supercritical environment only one phase exist. The fluid is neither a gas nor a liquid, and is best described as intermediate to the two extremes. Such fluids are called supercritical fluids (SCF). Supercritical fluids offer very efficient extraction characteristics, owing to its favourable diffusivity, viscosity, surface tension and other physical properties. Supercritical fluids retain solvent power approximating liquids as well as the transport properties common to gases. Supercritical fluid (SCF)

has a density closer to that of liquids, and its viscosity and surface tension are relatively low, which enable it to easily penetrate e.g. the botanical material from which the active component can be extracted. Diffusivity is one to two orders of magnitude higher than those of other liquids, which facilitates rapid mass transfer and faster completion of extraction than conventional liquid solvents. The gas-like characteristics of SCFs provide ideal conditions for extraction of solutes giving high degree of recovery in a short period of time. It also has the superior dissolving properties of liquid solvent and can selectively extract target compounds from complex mixtures *(Mukhopadhyay, 2000).*

The experimental techniques for measuring solubility of a heavy liquid or a solid in a supercritical fluid (SF) are either dynamic or static. In the dynamic mode, the solute is continually swept with fresh SF, mainly in fractionation or sequential studies.

In the static mode, both the solute and the solvent are loaded together into the extraction cell and held static for some fixed time *(Dean, 1998; Mukhopadhyay 2000)* .For some vegetable oil extraction procedures, an exhaustive extraction of the compounds is desirable. In these cases, the solubilizing power of the solvent should be maximized to achieve extraction of compounds with lower solubility, such as triglycerides.

The solubilizing power is a function of the supercritical fluid density and depends on both the pressure and temperature. The capability to control the density, temperature, and fluid composition allows fractionation of the sample analytes to be selectively removed in a series of steps. The polarity of SF can be changed by the addition of small amounts of organic solvents (modifiers) to supercritical CO_2. Changes in the type of modifiers for supercritical CO_2 allow the selective removal of analytes from the matrix in more than one fraction. This procedure is also a fractional technique and is called ‘sequential extraction’, multi-step extraction or multiple extractions. In summary, when the same modifier is used and only the quantity of modifier is changed, the procedure is called, “fractional extraction”, when different modifiers are used to modify the fluid composition the procedure is called “sequential” *(Tomasko et al., 1999).*

Partial extraction procedures, such as the extraction of essential oils, the initial CO_2 can not have the maximum solubilizating power due to the risk of solubilizing undesirable compounds. In these procedures, selectivity is the most important factor, and the indicated regions of pressure and temperature are those near the critical pressure and temperature *(Pellerin, 1991).*

The solvating power of an SF achieved with changes in the density when near its critical points can be better explored by fractional extraction procedures. In this way, the analytes can be removed selectively from the matrix in a series of fractions by changing the conditions of pressure, temperature, and fluid

polarity. Selectivity for supercritical extraction means that by using fractional procedures, it is possible to fractionate a sample into different compound classes such as essential oils, tritepenses, fatty acids, resins, pigments, etc. Sequential extraction seems to be the most interesting system for samples showing large quantities of nonvolatile products. However, few studies of fractional or sequential extraction with SFs and modifiers have been reported *(Tomasko et al., 1999).*

As Table 2 shows, the critical temperatures and pressures of gases and liquids can differ by hundreds of degrees; these differences suggest the use of specific supercritical fluids in specific applications. For example, because the critical temperatures of carbon dioxide, ethane and ethylene are near ambient, they are attractive solvents for processing heat-sensitive flavors, pharmaceuticals, labile lipids and reactive monomers.

Substances that are less temperature-sensitive, such as industrial chemicals and polymers, are readily treated with the C3 and C4 hydrocarbons with critical temperatures in the range of 100-150C°, they are generally better solvents for polymers than C2 hydrocarbons. Benzene and toluene with high critical temperatures of 250-300C°, are used to process non-volatile substances such as coal and high molecular weight petroleum fractions. Supercritical water with high critical temperature of 374,5C° is being used for hazardous waste detoxification and hydrocarbon reforming *(McHugh and Krukonis, 1994).*

The most desirable SCF solvent for extraction of natural products for foods and medicines today is carbon dioxide (CO_2). It is an inert, inexpensive, easily available, odorless, tasteless, environment-friendly and GRAS (generally regarded as safe) solvent. Further, in SCF processing with CO_2, there is no solvent residue in the extract, since it is a gas in atmospheric condition. In addition, its near-ambient critical temperature (31.1C°) makes it ideally suitable for thermo labile natural products. Due to its low latent heat of vaporization, low energy input is required for the extract separation system, which renders the most natural smelling and natural tasting extracts. Due to the relatively high applicable pressure, the investment cost of such a plant is higher than that of the conventional units, although the process is easily controlled with low operating costs.

Further, the energy required for attaining supercritical state of CO_2 is often less than the energy associated with distillation of conventional organic solvent. In general, the extractability of the compounds with $scCO_2$ depends on the occurrence of the individual functional groups in these compounds, their molecular weights, and polarity.

Table 2. Critical conditions for various supercritical solvents (McHugh and Krukonis,1994; Mukhopadhyay, 2000).

Fluid	Normal boiling point (°C)	Critical constants		
		Temperature (°C)	Pressure (bar)	Density (g/cm^3)
Carbon dioxide	-78.5	31.1	73.8	0.468
Ethane	-88.0	32.2	48.8	0.203
Ethylene	-103.7	9.3	50.4	0.200
Propane	-44.5	96.7	42.5	0.220
Propylene	-47.7	91.9	46.2	0.230
Chlorotrifluoro-methane	-81.4	28.9	39.2	0.580
Trichlorofluoro-methane	23.7	198.1	44.1	0.554
Nitrous oxide	-89.0	36.5	71.0	0.457
Ammonia	-33.4	132.5	112.8	0.240
Water	100.0	374.2	220.5	0.272
Benzene	80.1	289.0	48.9	0.302
Toluene	110.0	318.6	41.1	0.290

For example, hydrocarbons and other organic compounds of relatively low polarity are extractable in $scCO_2$ at a lower pressure in the range of 75 to 100 bar, whereas higher pressure needed for extraction of substituted terpenes, sesquiterpenes, lipids, carotenoids and waxes. The highly polar compounds such as the ones with one carboxylic and three or more hydroxyl groups are scarcely soluble. For the extraction of low soluble compounds, a co solvent or an entrainer is often applied to increase the polarity and hence the solvent power of $scCO_2$. Ethanol, ethyl acetate and possibly water are the best natural entrainers for food-grade products. Without causing additional ″ green house effect″ commercial CO_2 is available from environmental system, obtained as by-product from the fermentation process or the fertilizer industry. Moreover, there is also the possibility for continuous process where the $scCO_2$ after extraction is liquefied and recycled to the beginning of the process *(McHugh and Krukonis, 1994; Simándi et al., 1996 ; Oszagyán et al., 2000 ; Simándi et al., 2000 ; Gamse, 2000 ; Mukhopadhyay, 2000).*

2.1.2. *Thermodynamics of supercritical fluid state*

Thermodynamically, SCF is a state where the pressure and temperature are beyond the critical point values. If we look the pressure – temperature phase diagram of CO_2 (Figure 2) in supercritical fluid state (above 31.1°C and 73.8 bar) liquid-like densities (ρ) can be found. Also the dielectric constant (ε) show similar trends with pressure as well as the variation of density. Both ρ and ε rise sharply between

70 and 200 bar, while around 200 bar and beyond, both parameters attain values similar to those for liquids.

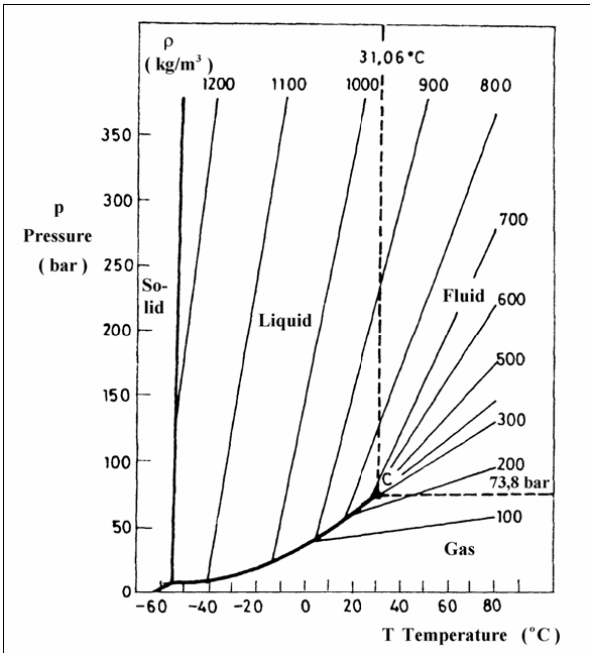


Figure 2 P-T digram of CO_2 at densities from 100 to 1200 kg/m³ (McHugh and Krukonis, 1994).

2.1.3. *Solubility of solids in supercritical fluids*

The basics for using a supercritical fluid as extraction medium are the solubility and phase equilibrium of substances in the compressed gas. The solubility and phase equilibria can be varied in a wide range by changing pressure and temperature. Figure 3 shows a typically trend of extraction yield over extraction time. At the beginning extraction efficiency is limited by solubility in the available amount of fluid. Higher solubilities and therefore shorter extraction times can be achieved by increasing extraction pressure, because with higher fluid density also solvent power increases, and normally by increasing extraction temperature, because at higher pressure levels the increase in vapor pressure of the substances to be solved is more efficient than the decrease of fluid density at higher temperatures. The second phase of extraction is controlled by diffusion and especially this phase causes long extraction times. Therefore, it is the aim to reach the proposed extraction yield within the solubility phase because otherwise the process will not be economically accepted *(Gamse, 2002)*.

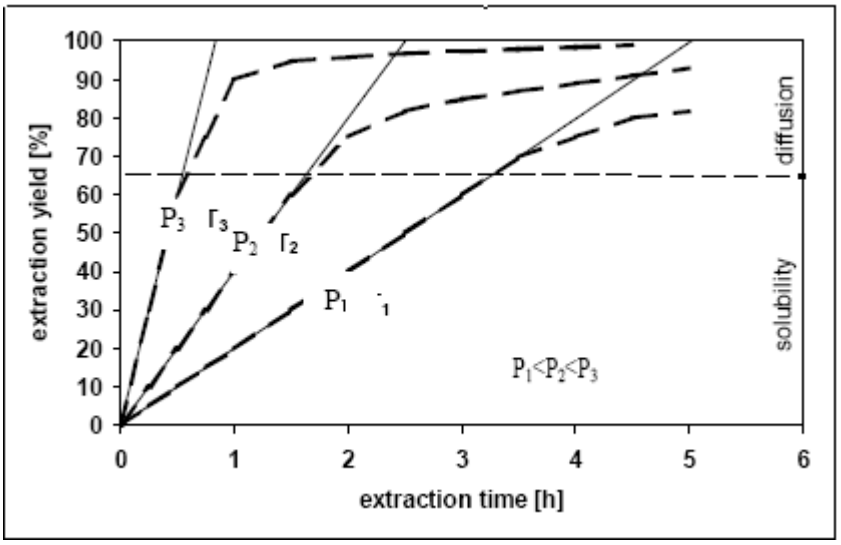


Figure 3.. Typical extraction curves by SFE (Gamse, 2002).

2.1.4. *Transport properties of SCF*

Successful design and development of a supercritical fluid extraction (SFE) process plant on the commercial scale while retaining its economic feasibility rely not only on the knowledge of the unique solvent characteristics, but also on the understanding of transport properties of scCO2. Three principal transport properties of industrial interest are the viscosity (η), the diffusivity (D), and the thermal conductivity (λ), which characterize the dynamics of the SFE process, involving themomentum transport related to the pressure difference, the mass transport related to the concentration difference, and the heat transport related to the temperature difference, respectively. The behaviour of these transport properties in the vicinity of the critical point is not fully understood due to very rapid changes in their values with respect to a small change in any one of the thermodynamic state variables *(Mukhopadhyay, 2000).*

2.1.4.1. *Viscosity*

The effect of pressure on the viscosity for a gas depends on the region of P and T of interest relative to the critical point. Near the critical state, the change in viscosity with T at constant pressure can be very large. The correlation of Uyehara and Watson is presented for the reduced viscosity estimated from the corresponding-states method. If viscosity graph is available, the reduced viscosity can be calculated. If the graph is not available, the critical viscosity can be estimated from the following equation:

$$\eta_C = 7.70 \frac{(M)^{1/2} p_C^{2/3}}{T_C^{1/6}}$$

$\eta_r = \eta / \eta_C$

where M refers to the molecular weight (g/mol), TC refers to critical temperature (K), pC refers to critical pressure (bar), η_C refers to critical viscosity (μP).

Figure 4 shows the viscosity diagram of CO_2, where the viscosity tends to minimum at the critical state. Near the critical point, at constant pressure, there are two branches, one in which viscosity increases with increasing temperature (gas-like behaviour), and the other branch where the viscosity decreases with increasing temperature (liquid-like behaviour) *(Recasens, 2002).*

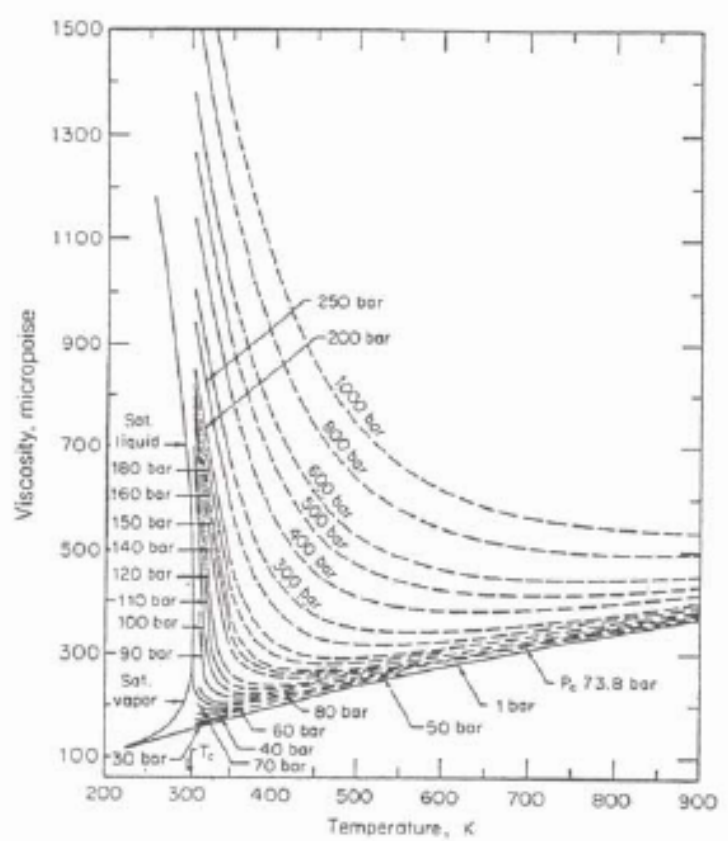


Figure 4. Viscosity of CO_2

2.1.4.2. *Diffusivity in dense gases*

For a binary mixture at a constant temperature and pressure, the molecular diffusion flux of a component (J_A) is defined by Fick's law as:

$$J_A = -D\frac{\partial C_A}{\partial z}$$

where D refers to the diffusivity constant (m^2/s), C_A refers to the molar concen
tration of A compound (mol/mol), z refers to the direction.

The diffusivity is a molecular parameter and is usually reported as an infinite-dilution, binary diffusion coefficient. Figure 5 shows the self diffusivity of CO_2 as a function of temperature over wide pressure range, which is approximately the same as the diffusivity of a molecule having a similar size diffusing through CO_2. The diffusivity in supercritical CO_2, in general, increases with temperature and decreases with pressure. At low pressure, the diffusivity is nearly independent of composition, whereas at higher densities, the composition dependence becomes more significant *(McHugh and Krukonis, 1994 ; Mukhopadhyay, 2000 ; Recasens, 2002).*

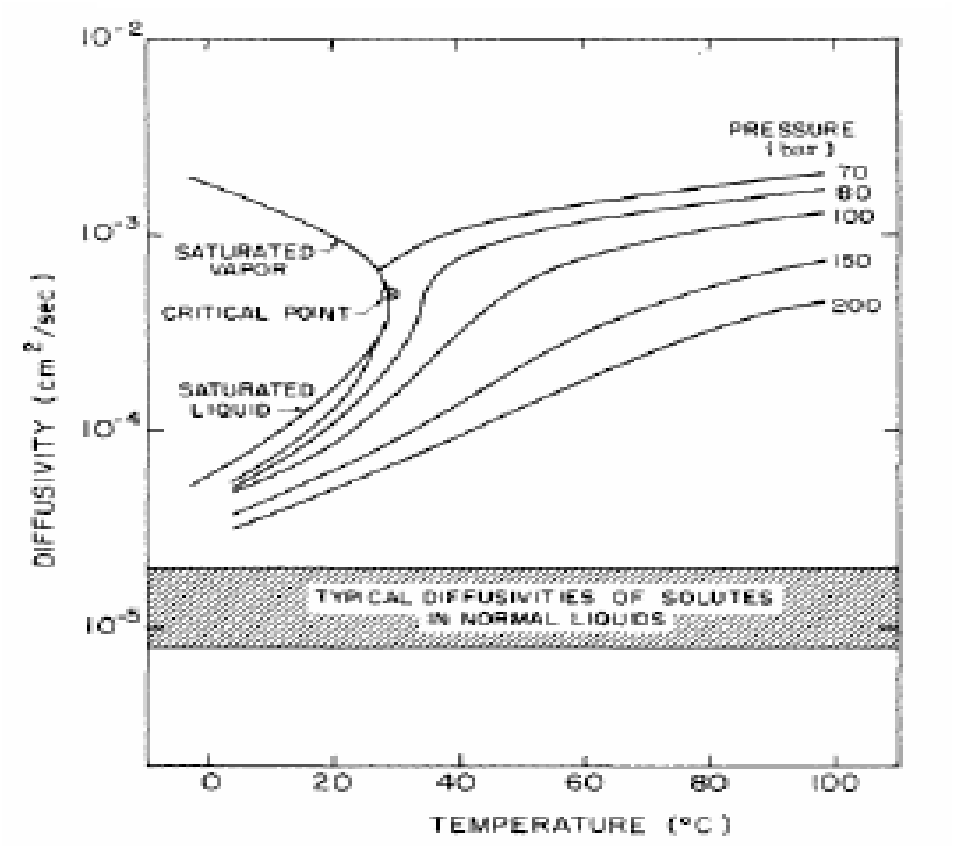


Figure 5. Diffusivity behaviour of CO_2 (McHugh and Krukonis, 1994).

2.1.4.3. *Thermal conductivity*

Thermal conductivity is defined as the proportionality constant of the linear relationship of heat flux with respect to temperature gradient as:

$$q = -\lambda \frac{\partial T}{\partial z}$$

where q is the heat flux and dT/dz is the temperature gradient. Thermal conductivity (λ) depends on temperature, pressure or density of the fluid. In general, thermal conductivity increases with increasing temperature and increasing density for most SCFs, as represented in Figure 6. On the other hand, at constant temperature, the thermal conductivity increases with pressure. Near the critical point relatively large values are reported (0.25-0.30 W/Km) *(Mukhopadhyay, 2000; Recasens, 2002).*

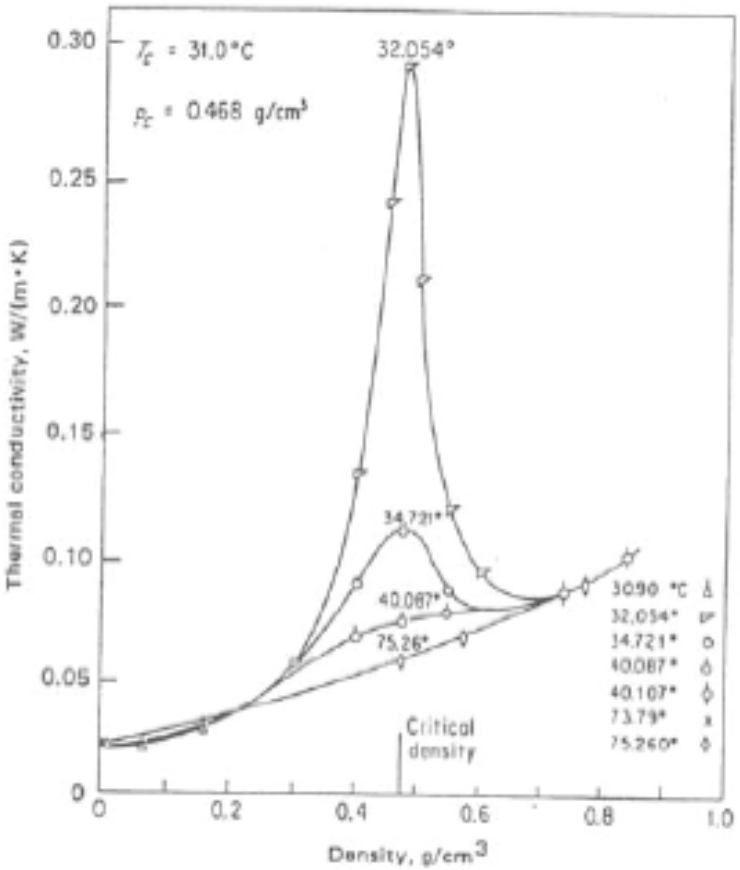


Figure 6. Thermal conductivity of CO_2 near critical point ((Recasens, 2002).

2.1.5. *Mass transfer behaviour*

Mass transfer in high pressure systems is related to the extraction of a valuable solute with a compressed gas. This is either a volatile liquid or solid deposited within a porous matrix. The compressed fluid is usually a high-pressure gas, often a supercritical fluid. In supercritical condition, the density of gas approaches a liquid-like value, so the solubility of the solute in the fluid can be substantially enhanced over its value at low pressure. The retention mechanism of the solute in the solid matrix is only physical (unbound, as with the free moisture), or strongly bound to the solid by some kind of link (as with the so-called bound moisture). Crushed vegetable seeds, for example, have a fraction of free, unbound oil that is readily extracted by the fluid, while the rest of the oil is strongly bound to cell walls and structures. This bound solute requires a larger effort to be transferred to the solvent phase *(Recasens, 2002).*

Depending on the mechanism of mass transfer, there might be up to three different regimes of extraction, namely (1) constant rate (solubility controlled) regime, (2) falling rate-phase I (diffusion controlled) regime, and (3) falling rate-phase II (desorption controlled) regime. When a fixed bed of solid is contacted with flowing CO_2 at a selected supercritical condition, the mass transport mechanism involves diffusion, and adsorption of SCF solvent followed by solute desorption, diffusion through pores, and the convective transport along with the flowing SCF solvent across the bed height. But the crucial factor is the initial distribution of the extractable substance within the solid substrate which may exist in the adsorbed state either on the outer surface or on the surface of pores, or may exist in the dissolved state in the cytoplasm or the vacuoles within the plant cells. The extraction process entails the following sequential and parallel steps at steady mode of extraction at the beginning of extraction:

1. Diffusion of CO_2 into the pores and adsorption of CO_2 on the solid surface;
2. Transport of oil to the outer layer and formation of a thin liquid film around the solid particles;
3. Dissolution of oil in $scCO_2$;
4. Convective transport of the solute to the bulk of the fluid.

Subsequently, at the unsteady mode of extraction, the SFE process entails:

5. Desorption of solute from the solid or pore surface followed by ;
6. Dissolution of the solute in $scCO_2$;
7. Diffusion of the solute in the pores;
8. Convective unsteady state transport of the solute to the bulk of the fluid.

If we think these steps through the step 4 seems to be the slowest one, when there is a liquid film of oil present on the outer surface of the solid particle and the mass transfer takes place at a constant rate depending on the solubility of oil in the solvent, and the mass transfer rate is controlled by the external film resistance *(Mukhopadhyay, 2000)*.

The nature of mass transfer kinetics depends on the amount, the localization of the solute also the strength of the bound of the solute to the matrix, the mechanism of its release from the substrate, and the nature of the solute transport within the solid matrix, besides other parameters like pressure, temperature, flow rate, particle size, etc.

The size of the solid particles is a crucial factor in deciding the nature of extraction kinetics, particularly for solids containing less solute. In general, extraction rate as well as yield increases with decreasing particle size, by reducing the path of transport within the solid and by breaking larger number of oil vacuoles to release more extractable compounds. However, the smaller particle size may result in a higher pressure drop and uneven distribution of particles, rendering a lowering of mass transfer rate *(Mukhopadhyay, 2000)*.

For optimization the SFE process experimental results and/or models are required. For example, Reverchon explained the mechanism of SFE from herbaceous matrix to produce essential oil and leaf and cuticular waxes that dissolution of these compounds are based on different mass transfer mechanisms. Cuticular waxes are mainly solubilized by leaching while essential oil extraction is supposed to be dependent on complex diffusion phenomena. Leaves of some plant families are characterized by epidermal hair, known as glandular trichomes, in which essential oil is accumulated. Moreover, small quantities of the essential oil, released from broken cells during drying and milling, can also be located near the particle surface. Leaf and cuticular waxes are located on the leaf epidermis, because they exert a controlling function on water exchange between leaves and air. At lower pressures (80-150 bar) and temperatures (35-50°C) the $scCO_2$ extractable part of culticular waxes is mainly constituted by n-alkanes ranging from C_{25} to C_{31}.

2.1.6. *Industrial applications*

Decaffeination of coffee and tea is the largest application for the supercritical fluid extraction. This is valid for the investment costs of the plant and for the capacities of the plant. The second largest application is the extraction of hop. In the last twenty years nearly all producers of hop extracts changed to the liquid or supercritical CO_2 extraction process. On the third place is a new industrial application with which the removal of the pesticides from cereal is carried out. The first plant started operation end of 1999 in Taiwan and since then two others have been built up in Far-East.

The extraction of spice oleoresins is relatively new and since fifteen years industrial plants are in operations. Due to the fact, that the CO_2 extracts are different to the conventional oleoresins the acceptance in the food industry is very slow. The spice plants are much smaller compared to the decaffeination plants and the hop plants. The size of the extractors is between 200 and 800 l. The same is valid for medicinal herbs and high value fats and oils, which are more or less at the beginning of the development *(Simándi and Sawinsky, 1996; Nakahara et al., 2000; Gamse, 2000; Simándi et al., 2000; Simándi and Sawinsky, 2002).*

Many research teams used supercritical carbon dioxide for oleoresin extraction from cardamom seeds and thyme leaves the in the last 15 years. Simándi and his team extracted oleoresin from wild thyme and made a comparison between thyme oil and supercritical carbon dioxide extract of hungarian wild thyme, where they found that both oils yielded qualitatively similar products (*Oszagyán et al., 1996a*). Also the same team extracted volatile compounds from lavandin and thyme(*Oszagyan et al., 1996b*). Moldao-Martins et al., (2000) used supercritical CO_2 for extraction of *thymus zygis L.subsp.sylvestris* aroma. Supercritical fluid chromatography-mass spectrometry of thyme extracts by (*Blum et al., 1997*). A comparative analysis of the oil and supercritical CO_2 extract of *Elettaria cardamomum (L.)* Maton was done by (*Marongio et al., 2004*) where they got a yield of 5.5% of oleoresin at 90 bar and 40°C and terpinyl acetate (42,3% and 1,8 cineol (21,4%) were the major volatiles.

Specific application processes

Beside the typically extraction purposes different applications using supercritical fluids, mainly carbon dioxide, are tested as well in laboratory as in industrial scale.

Decontamination of soils using supercritical fluids is an attractive process compared to extraction with liquid solvents because no toxic residue is left in the remediated soil and in contrast to thermal desorption soils are not burned. Especially removal of typically industrial wastes like PAHs, PCBs and fuels can be removed easily. The main applications are preparation for analytic purposes, where supercritical fluid extraction acts as a concentration step which is much faster and cheaper than solvent extraction. Main parameters for successful extraction are water content of the soil, type of soil and contaminating substances, available particle size distribution and the content of plant material, which can act as adsorbent material and therefore prolong extraction time.

The ***neutralization and impregnation of paper*** is of great interest for all libraries, because acid degradation results in a decrease in the pH and weakening of the mechanical properties of the paper. For this reason in the first step the degradation products are extracted with supercritical carbon dioxide

which increases the pH. In the following step neutralizing substances are solved in the CO_2 stream and impregnation of the paper takes place.

Supercritical CO_2 is used in a process for ***bone tissue treatment*** to obtain a novel bone substitute for human surgery. The supercritical extraction step results in delipidation of bones and therefore decreases of infection effects. Then efficient enzymatic deproteination can be performed.

In the field of ***polymer recycling*** and/or disposal new techniques are highly required. Most of the electronic waste contains flame retardants, mainly halogenated organic substances. In many recycling processes plastics are incinerated and the formation of halogenated dibenzodioxins and dibenzofurans cannot be avoided. One promising way to separate halogenated flame retardants from polymer matrices is the extraction with supercritical carbon dioxide. The advantage of this process is that as well the polymer as the flame retardant can be recycled, especially because flame retardants are relatively high price products*(Simándi and Sawinsky, 1996; Bruneton, 1999; Simándi et al., 2000; Nakahara et al.,2000; Sovova et al.,2001).*

2.2. Herbs and medicinal plants

Hippocrates (5th century B.C.) mentioned 300 to 400 medicinal plants. Nowadays around 18 000 species of plants have been used in food and in drugs *(Bruneton, 1999)* . Herb extracts have been applied thousand of years with Persian, Egyptian and Indian origins. The herb extracts are taken from plants in many different ways; expression, enfleurage, maceration, solvent extraction, and distillation

2.2.1. *Wild Thyme* (*Thymus Serpyllum L.*)

Thymus serpyllum L. *Thymus vulgaris L.*

Figure 7. Thyme

2.2.1.1. Botany

The genus Thymus includes about 350 species world-wide and distributed mainly in temperate.

Thymus serpyllum L. belongs to *Lamiaceae* family. Thyme is an aromatic plant and spice that originates in the Mediterranean region and India *(Lee et al., 2004).* The garden thyme is a cultivated form of wild thyme (*Thymus serpyllum L.*) which is also found in the Hungarian wild. Wild Thyme or Creeping Thyme (*Thymus serpyllum*) is a species of thyme native to most of Europe , North Africa and Middle East. It is a low, usually prostrate subshrub growing to 2 cm tall with creeping stems up to 10 cm long, with oval evergreen leaves 3-8 mm long. The strongly scented flowers are either lilac, pink-purple, magenta, or a rare white, all 4-6 mm long and produced in clusters. The hardy plant tolerates some pedestrian traffic and produces odors ranging from heavily herbal to lightly lemon, depending on the plant. *(Simon et al., 1984; Chevallier, 1996*; *Bruneton, 1999; Gersbach et al.,2001).*

A combination of dried wild Palestinian Thyme leaves, salt, sesame seeds and the fruits of the tree *Rhus coriaria* are called " Zaatar " in Arabic, a very popular mixture that is used almost daily in the Middle East as food, additive in salads and spice for pastry and meat.

2.2.1.2. Chemical composition

Thyme is one of the oldest medicinal plants used in folk medicine for a wide range of ailments. Its essential oil have been officinal since the sixteenth century, it is first enumerated in the Dispensatorium Noricum of 1589. Thyme drog(*Thymi herba*) contains 1-2.5% of essential oil (two main chemotypes exist), tannins, flavonoids,caffeic acid, ursolic acid and oleanolic acid *(Leung and Foster, 1996; Bruneton, 1999 ; Bernáth, ,2000).* The hydrodistillated essential oil has dark, reddish-brown, pleasant, strong spicy-phenolic odor and biting, persistent taste *(Bauer et al., 1990).* Among the two chemotypes the phenolic type dominates, its main constituents of essential oil are carvacrol and its isomer thymol (up to 70%), p-cymene (~15%), pinene, mentene, borneol (~15%), linalool (~15%) and cineole. The oil representing the other chemotype, contains mainly geraniol, linalool, α-terpineol and cineole; these oils are of minor importance (*(Bauer et al., 1990).* The thymol as main component was observed in 1719, therefore belongs to those compounds from volatile oils longest known. Thyme essential oil contains mainly the same compounds although some differences can be recognised due to the geographic differences. Kulisic found the following composition in *Thymus serpyllum*: α-pinene (1%), β-myrcene (0.5%), γ-terpinene (1.18%), p-cymene (2.99%), borneol (1.65%), terpineol (0.20%), caryophyllene (1.16%), carvacrol (90.8%) (*Kulisic et al.,2005*). Recently the composition of Albanian thyme essential oil was revealed with the main components: p-cymene (7.76-43.75%), γ-terpinene (4.20-27.62%), thymol (21.38-60.15%), carvacrol (1.15-3.04%) and β-caryophyllene (1.30-3.07%) *(Asllani and*

Toska, 2003). In 1996 the essential oil composition of a Spanish wild growing plant was described with 1,8-cineole and linalool as main components *(Guillen and Manzanos,,1998 a)*. *Oszagyán et al.,* (*1996a*) found the following volatiles in the hydrodistilled Hungarian wild thyme oil, collected from Transdanubia area of Hungary : ρ-cymene (7.96), linalool (5.62), borneol (2.76), terpinen-4-ol (1.95), α-terpineol (2.38), nerol (6.96), thymol (9.35), carvacrol (45.94), geranyl acetate (2.86), β-caryophyllene (2.46), and β-bisabolene (2.73). A quantitative comparison of constituents present in the supercritical fractional extract and in the hydrodistilled wild thyme oil was also made by Oszagyán research team giving qualitatively similar products, and carvacrol and thymol were the main components (*Oszagyán et al.,1996a).* Wild thyme collected from different areas of Hungary resulted some differences in composition of their oils. The result showed that the environmental conditions may influence the quantitative composition of wild thyme (*Oszagyán et al.,1996a).* Also leaves of wild thyme collected from different part of Palestine gave different thymol, carvacrol concentration (*Abu-Lafi et al., 2007*). Production of thymol or carvacrol seems to depend on some external variation such as the soil, climate conditions, harvesting time and the amount of water to which the herb is exposed. Apparently these factors favor the formation ofone isomer over the other (*Kimura et al., 2006*).

The identified volatiles of palestinian wild thyme were maily: o-cymene (12.1 %), γ-terpinene (25.86%), thymol (10.48%),and carvacrol (7%). Palestinian thyme harvested in January and February contained thymoquinone (1.96) while thyme harvested in the next months showed traces (*Abu-Lafi et al., 2007*).

Generally, the yield of essential oil obtained from flowers and leaves are higher than those from stem or other woody part. The compositions of these essential oils were different too. From terpene and sesquiterpene hydrocarbons (HC) and their oxygenated compounds, the leaves contained the most (449 mg/kg for terpene HC), the flowers (162 mg/kg for terpene HC), while the stem contained the less (0.7 mg/kg for terpene HC). From phytosterols the stem contained the least (80.4 mg/kg) in comparison with flowers (157 mg/kg) and with leaves (201 mg/kg), respectively (*Guillen and Manzanos,1998B*). Haraguchi et al.(1996) isolated a biphenyl compound, 3,4,3′,4′-tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl and a flavonoid, eriodictyol and revealed that the biphenyl compound was strong antioxidant *(Marino et al., 1999).* In 1998 other flavonoid glycosides were isolated from butanol-soluble fraction of thyme. Among those flavonoids,eriodictyol-7-rutinoside and luteolin-7-O-β-glucopyranoside showed the strongest antioxidant properties *(Hudaib et al., 2002).* Same research group identified from the same fraction of thyme four acetophenone glycosides (picein and androsin) with two new compounds. The compounds showed relatively weak cytotoxicity on human leukemia cell HL-60 *(Schwarz et al., 1996).* Anti-complementary polysaccharide (TV-3-IIIA-IIa) was identified

from hot-water extract of thyme leaves with the presences of arabinogalactan and pectin-like polysaccharide *(Haraguchi et al., 1996)*. High content of rosmarinic acid (91.8 mg/100 g fresh weight), luteolin (39.5 mg/100 g fresh weight), hispidulin and caffeic acid in smaller amounts were quantified in the extract of thyme *(Wang et al., 1998)*. In the next year four other flavonoids were identified from thyme and the antioxidant activity of these compounds were measured by the oil stability index (OSI) method. Among diterpenes, carnosol, isorosmanol, carnosic acid, rosmanol, epirosmanol and galdosol exhibited strong antioxidant activities measured with OSI method in methyl linoleate system *(Miura et al., 2002 ; Wang et al., 1999)*. Strong radical scavenger compounds (rosmarinic acid, eriodictyol, taxifolin, luteolin-7-glucuronide, p-cymene-2,3-diol, p-cymene-2,3-diol 6-6'-dimer, carvacrol and thymol) were isolated from leaf extract of thyme by Dapkevicius et al. *(Dapkevicius et al., 2002)*. The main phenolic and essential oil compounds can be seen in (Figures 8, 9).

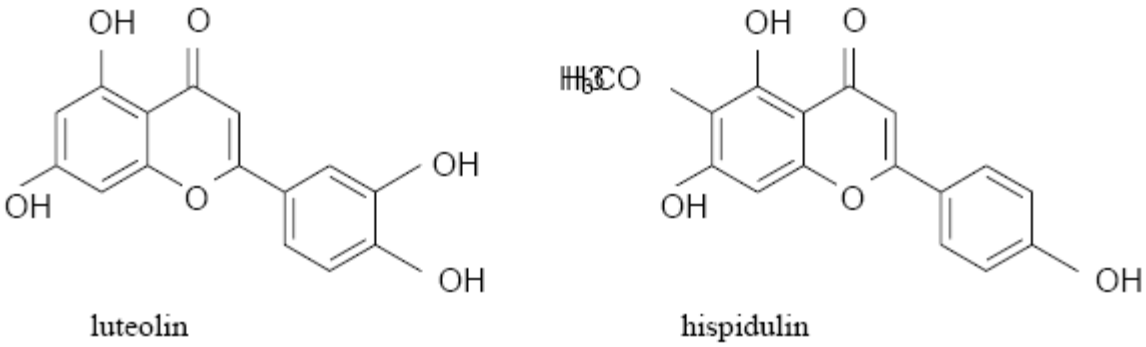


Figure 8. Main flavonoid compounds in Thymus sepyllum L.

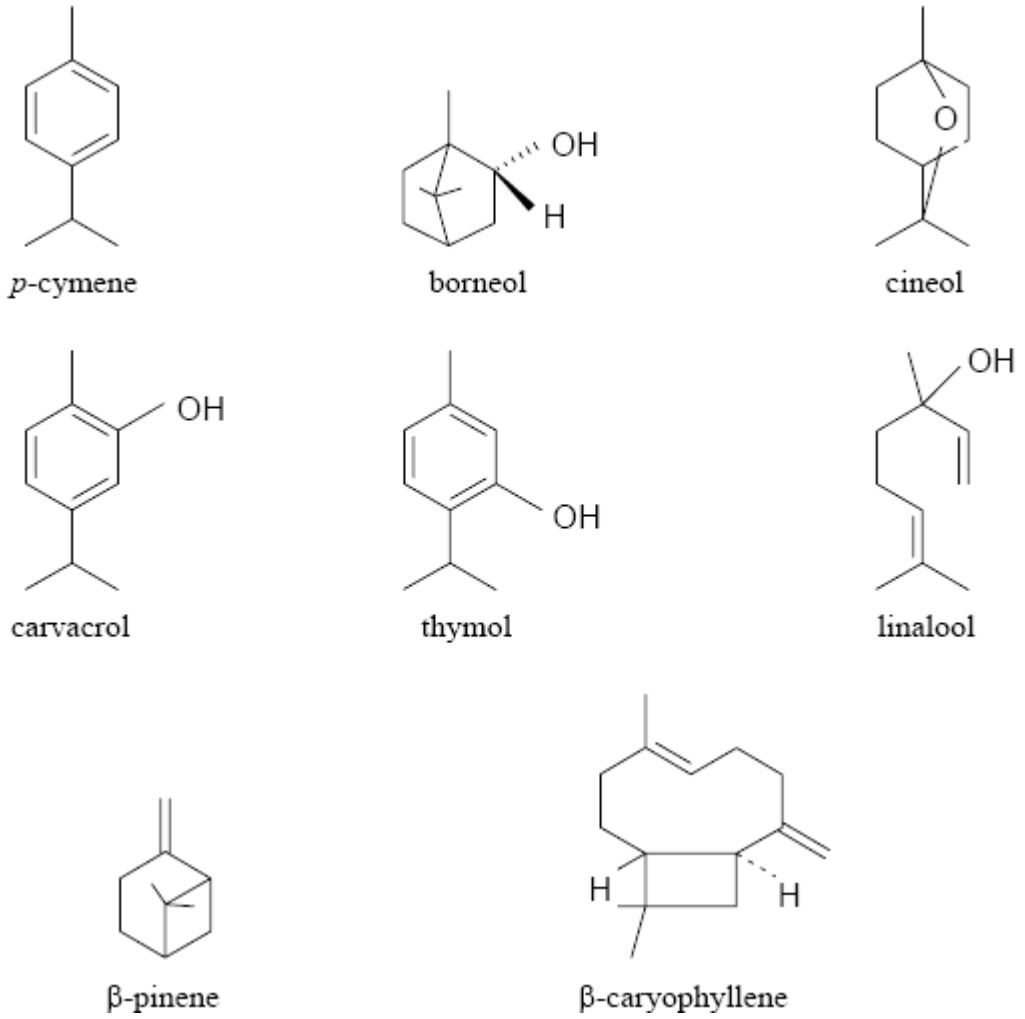


Figure 9. Essential oil compounds of Thymus serpyllum L.

2.2.1.3. Ethnopharmacology

As a medicinal plant, thyme has traditionally been considered an anthelmintic, antispasmodic, carminative, expectorant, disinfectant, deodorant, sedative, stimulant and tonic. The plant is used internally in the treatment of dry coughs, whooping cough, bronchitis, bronchial catarrh, asthma, indigestion, gastritis and diarrhoea. Externally, it is used in the treatment of tonsillitis, gum diseases, rheumatism, arthritis and fungal infections. Thyme has been also used to promote perspiration *(Simon et al., 1984; Price, 1995; Leung and Foster, 1996 ; Blumenthal, 1998).* Numerous reports can be found to reveal the antimicrobial activity of thyme herb essential oil or the main compounds, as thymol and carvacrol. The antibacterial tests are mainly carried out against foodborne and human pathogen bacteria. Among herbs, thyme has shown the strongest antibacterial activity against Gram-positive

(e.g.: *Staphylococcus aureus, Bacillus licheniformis*, *Listeria monocytogenes*) and Gram-negative (e.g.*: Escherichia coli, Pseudomonas fluorescens*, *Yersinia enterocolitica, Salmonella typhimurium*) bacteria *(Chun et al., 2001; Essawi and Srour, 2000; Leung and Foster, 1996)*. Smith-Palmer et al. (1998) mentioned that Gram-positive bacteria showed more sensitivity to inhibition by plant essential oils than the Gram-negative bacteria However, *Pseudomonas aeruginosa* showed less sensitiveness, inhibition was observed only.at higher concentration (500 µg/ml) *(Paster et al., 1990).* Thyme essential oil (up to 0.33% concentration) strongly controlled of *Botrytis cinerea* leaf colonisation and bunch rot in grapes *(Walter et al., 2001).* In antifungal tests mainly mycotoxigenic and crop attacking phytopathogenic fungi have been examined. Thyme essential oil was applied against mycelia and spores of *Aspergillus niger, Aspergillus flavus* and *Aspergillus ochraceus* as well as against natural microflora of wheat grains *(Paster et al., 1995; Montes-Belmont and Carvajal, 1998).* Thyme essential oil (in <500 ppm) showed complete inhibition on test fungi against the above mentioned pathogens and *Fusarium moniliforme* (*Soliman and Badeaa, 2002*). Thyme essential oil inhibited at relatively low concentrations (250-400 µg/ml) a postharvest pathogen (*Penicillium digitatum*), other food pathogen fungi (e.g. *Trichoderma viride, Microsporum gypseum, Fusarium solani*), and thyme oil showed antifungal activity against human pathogens as *Candida albicans* and *Trichophyton mentagrophytes (Zambonelli et al., 1996 ; Zollo et al., 1998 ; Daferea et al., 2000; Giamperi et al., 2002***).** The antimicrobial properties of thyme essential oils are maily related to their phenolic content (*Dimitrijevic et al., 2007)*. Thyme oil due to its high phenolic compounds demonstrated great sublethal effects on the tobacco cutworm (*Spodoptera litura*) *(Hummelbrunner and Isman, 2001).* The antifungal and antiaflatoxigenic properties of thyme essential oil constituents were revealed and thymol completely inhibited *Aspergillus* flavus growth and aflatoxin formation *(Mahmoud, 1994).* On the other hand, regarding to the research by Roller and Seedhar, carvacrol (in 1 mM concentration) delayed the spoilage of fresh-cut kiwifruit and honeydew melon at chill temperature without adverse sensory consequences *(Roller and Seedhar, 2002).*

The strong antioxidant activity of thyme leaf extracts and essential oils containing phenolic compounds, flavonoids and tannins have been summarized by several authors *(Wang et al.,1998 ; Wang et al., 1999 ; Miura et al., 2002).* Dapkevicius and co-workers pointed out that acetone extract of thyme showed higher antioxidant activity than extracts obtained by apolar solvents *(Dapkevicius et al., 1998)*.The antioxidant properties of thyme and wild thyme eesential oils have been studied by (*Kulisic et al., 2005*) and showed that these oil have an antioxidant activity but thyme is slightly better than wild thyme. A research group proved that the antioxidant activity of an extract cannot be predicted on the basis of its total phenolic content. Thyme essential oil exhibited high antioxidant activity due to not

only the phenolic compounds but to flavonoid and to the contradiction of all presented compounds (*Schwarz, 1996*). According to Simándi research group the antioxidant activity of thyme supercritical and ethanolic extracts were substantially measured by Rancimat method (*Simándi et al., 2001*). In lipophilic systems, such as in the aldehyde/carboxylic acid assay and in the conjugated diene assay thyme extracts exhibited strong antioxidant activity (*Lee and Shibamoto, 2002*). De-odourised aqueous extract of thyme showed considerably OH• radical scavenging activity due to the rosmarinic acid present in the aqueous extract, although the total phenolic content of thyme extract was the lowest among the examined *Lamiaceae* plants (*Dorman et al., 2003).*

2.2.1.4. Uses

Thyme is used most widely in cooking. Thyme is a basic ingredient in French and Italian cuisines, and in those derived from them. It is also widely used in Arabian and Caribbean cuisines.Thyme herb is used in raw in salads and for flavouring cheeses, soups, stews, stuffings, meats, fishes, dressings, sauces and honey. Leaves and flowering tops are used in sachets and also used to repel moths from clothing. The thyme tea cures coughing, sore throat, fever and the symptoms of cold and flu *(Simon et al., 1984 ; Leung and Foster, 1996).*

In some Middle Eastern countries, the condiment *za'atar* contains thyme as a vital ingredient.

Thyme essential oil is used mainly for flavouring foods and oral hygiene products (toothpaste, mouthwashes, cough medicines), but is also used in perfumery to create spicy, leathery notes. Due to germicidal and antiseptic properties, it can be used in natural germicid products *(Simon et al., 1984 ; Bauer et al., 1990; Leung and Foster, 1996).*

2.2.2. Cardamom (Elettaria cardamomum)

Figure 10. Cardamom seeds

2.2.2.1. Botany

The cardamom of commerce is the dried ripe fruit (capsules) of cardamom plant. This is often referred as the "Queen of Spicees" because of its very pleasant aroma and taste, and is highly valued from ancient times. It is grown extensively in the hilly regions of South India at elevations of 800-1300 m as an under crop in forest lands. Cardamom is also grown in Sri lanka, Papua New Guinea, Tanzania and Guatemala.

Cardamom belongs to the genus *Ellettaria*, and species *Cardamomum* (Maton). The genus name is drieved from the Tamil root *Elettaria*, meaning cardamom seeds. The genus consists of about six species (*Mabberly, 1987*). Only *E. Cardamomum* Maton occurs in India, and the only economically important species. Cardamom grows to about 10 feet (3m) long. It has large leaves and white flowers with blue stripes and yellow borders. The fruit is a small capsule with 8 to 16 brown seeds. The seeds are found in ovalshaped fruit pods that are between ¼ and 1 inch long. The perennial herbs takes 3-4 years to start bearing the yellow-grey-capsules containing many seeds.*(Mathai, 1985)*. Fruits are gathered just before they are ripe in order to conserve the seeds inside the capsule, and then distilled to obtain the essential oil with an average yield from 2% to 5% *(Mehra, 2001.* The seeds are used as a spice and the plant itself is a perennial herb.

Cardamom has a checkered history, dating back to the Vedic period (Ca 3000 BC) and is among the ingredients poured into the sacrificial fire during the Hindu marriage.

2.2.2.2. Chemical composition

Dried fruit of cardamom contains steam-volatile oil, fixed (fatty) oil, pigments,proteins, cellulose, pentosans, sugars, starch, silica, calcium oxalate and minerals. The major constituent of the seed is starch (up to 50%) while in the fruit husk it is crude fibre (up to 31%). The constituents of the spice differ among varieties, variation in environmental conditions of growth, harvesting, drying procedures and subsequent duration as well as conditions of storage.The main factor that determines the quality of cardamom is the content and composition of volatile oil, which governs the odour and flavour.

Essential oil of cardamom is the source for its aroma and flavour. Research so far carried out concerned mainly with the composition of the oil. The characteristic odour and flavour of cardamom is determined by the relative composition of the components of volatile oil. Cardamom oleoresin contains about 52-58% volatile oil (*Purseglove et al., 1981*).

The first detailed analysis of cardamom oil was reported by Nigam et al., (1965) and the constituents were identified with the help of gas chromatography (GC) and infra red (IR) spectroscopy, using authentic reference compounds and published data. Ikeda et al., (1962) reported 23.3 % of the oil as hydrocarbons with limonene as a major component. They have also reported the presence of methyl heprenone, linalool, linalyl acetate, β-terpineol, geraniol, nerol, neryl acetate and nerolidol. Govindarajan et al., (1982), has elaborated the range of concentration of major falvour constituents, their flavour description and their effect on flavour use. The composition of volatile oils of cardamom from different sources measured by Govindarajan group where the following: α-pinene (1.4%), sabinene (3.2%), D-limonene (2.4%), 1,8 cineole (41%), linalool (0.4%), linyl acetate (1.6%), nerol (1.4%), α-terpinyl acetate (30%), methy heptenose (1.2%), neryl acetate (1.1%).

Most liquid food flavouring are volatile and chemically unstable in the presence of air, light, moisture and high temperatures. Such is the case for cardamom essential oil. Many herbs and spices, usually used to flavour dishes, are an excellent source of phenolic compounds *(Rice-Evans et al.,1996).* The main consistuents of essential oil in cardamom found by De Pradier were 1,8cineole (30.50%), sabinene (4.49%), linalyl acetate (4.4%), linalool (3.36%), limonene (3.12%), mycrene (2.54%), α-pinene (1.86%), α-terpineol (1.84%), α-terpinyl acetate (1.84%), terpinen-4-ol (1.14%), α-thujene, camphene, β-pinene, α-terpinene, cis-β-ocimene, γ-terpinene, trans-β-ocimene, p- cymene, terpinolene, sabinene hydrate, neral, geranial,geraniol, geranyl acetate and nerolidol in under 1% concentration (*De Pradier.E, 2006*). Cardamom oil is produced commercially by steam distillation of powdered fruits.

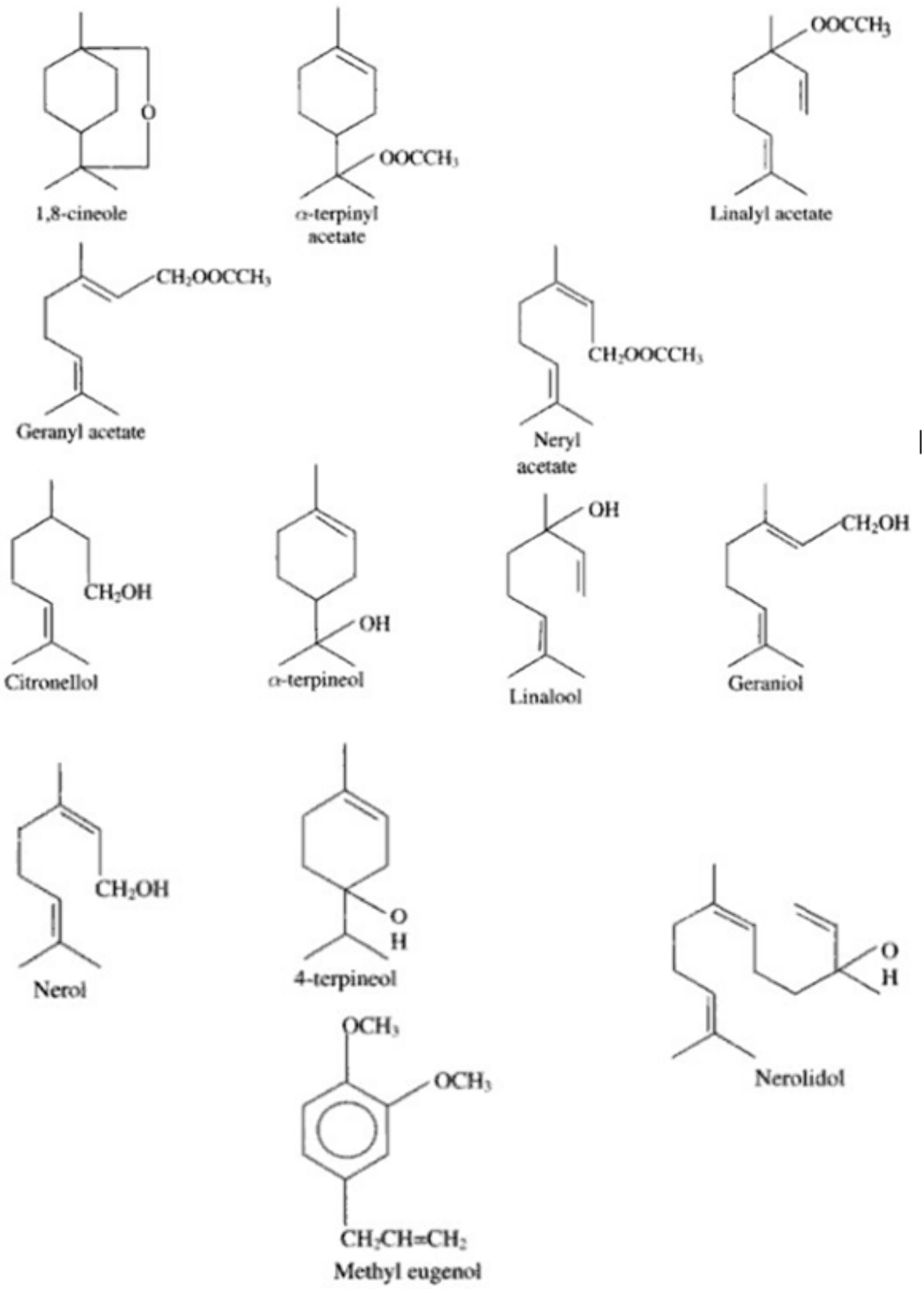


Figure 11.Structural formulae of major components of cardamom essential oil

2.2.2.3. Ethnopharmcology

Cardamom is tonic, eupeptic, carminative and appetizing. According to Franchomme and Pénoël *(Franchomme and Pénoél, 1990)* it is strongly tonic and stimulant, stomachic , and carminative. It is also strongly anticatarrhal and expectorant. To a lesser degree it is also listed as a neuromuscular antispasmodic. Studies have demonstrated that the essential oil has powerful spasmolytic effect via the blocking of muscarinic receptors to acetylcholine and that it is anti-inflammatory and analgesic *(Al-Zuhair et al., 1996*). The seeds are also prescribed in Ayurvedic medicine for coughs,cold, bronchitis, and asthma. Furthermore, according to some researchers,cardamom oil seems to have antibacterial, antiseptic, carminative and antispasmodic properties (*Al-Zuhair et al., 1996*). Moreover,some studies have considered the potential effects of essential oils from spices, including cardamom, against insect pests, including stored-product insects *(Huang et al., 2000)*. Cardamom oil is shown to have antibacterial and antifungal actions. Badei et al., (1991) studied the chemical composition, physiocochemical properties and antimicrobial activity of dried fruit of cardamom to asses the potential usefulness of cardamom oil as a food preservative. The antimicrobial effect of the oil was tested against 9 bacteria strains, 1 fungus and 1 yeast, the oil was 28.9 % as effective as phenol.

Cardamom oil is effective as an antioxidant for cottonseed oil as assessed by stability, peoxide number, refractive index, specific gravity, and rancid odour.

2.2.2.4 Uses

Cardamom is used as flavouring material in three forms; whole, decorticated seeds and ground. The spice distilledd for essential oil and solvent extraction for oleoresin. In the international trade generally whole cardamom is the item of commerce, while trade in decorticated seed is small and that of ground spice negligible. Cardamom is a principal ingredient in curry powders, and is used to flavor confectioneries, liqueurs and chewing gums. In some parts of the world, especially the Near East and Saudi Arabia, cardamom is ground and mixed with coffee. In southeast Asia cardamom is mixed with betel leaves for chewing. In the West it is used in perfumes. In Mexico and Guatemala you can buy cardamom chewing gum - it is delicious! Scandinavians use more cardamom than anyone else in the Western Hemisphere. They use it to flavor breads, cakes, candies, sausages and other meats. Add some ground cardamom to your next sweet pastry or apple pie.

Cardamom is also used in cosmetics and perfume industries. Medically, they are used for flatulent indigestion, and to stimulate the appetite (*Al-Zuhair et al., 1996.*

2.2.3. Antioxidant properties of medicinal herbs and extracts

Antioxidant properties are among the first links between chemical reactions and biological activitiy and have been extensively studied for the past 10 years. Many molecules, such as phenolic compounds, are well known to possess this activity as well as other biological activity, including DNA protective effects, enzyme inhibition, and prevention of cardiovascular diseases and cancer *(Kris-Etherton et al., 2002 ; Trouillas et al., 2003*). An antioxidant is a substance that when present at a concentration low compared to that of an oxidisable substrate, significantly delays or prevents oxidation of that substrate *(Rauha, 2001).* The application of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) is restricted in several countries, due to their possible carcinogenic effects proved with in vivo experiments *(Ribeiro et al., 2001 ; Anisimov, 2001).* Therefore, the natural antioxidant complexes, extracts and/or individual natural compounds are highly demanded within the food, cosmetic and pharmaceutical industries for health preventing and shelf life extending purposes. Aromatic plants have been studied extensively because a rich source of natural antioxidants is available in their essential oils or diverse extracts. Shahidi et al. (1992) reported that the antioxidant effect of aromatic plants is closely related to the presence of hydroxyl groups in phenolic compouds. Here are some bioactive compounds that possess antioxidant properties.

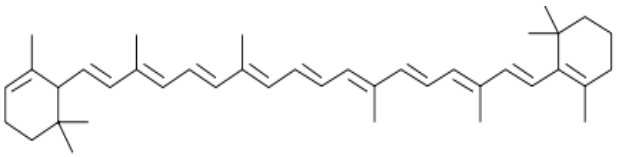
2.2.3.1. *Fat-soluble pigments*

2.2.3.1.1. Carotenoids

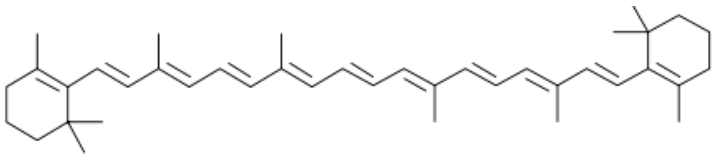
Carotenoids represent the major dietary source of vitamin A, and chlorophylls responsible for the attractive color of many foods. Carotenoids are pigments of yellow, orange-red color, occurring in both the flora and the fauna. Carotenoids are found in leaves of high plants together with, in other parts of plants (e.g. roots, fruits), and in microorganisms. In many cases they are the major pigments in the exoskeleton in of aquatic and avian species *(Sies, 1991)*. They are very widespread and occur naturally in large quantities. *Isler (1971*) has estimated that over 100.000.000 tons of carotenoids are produced annually by nature.

Carotenoids are a group of mainly lipid-soluble compounds. They have been classified into two major groups on the basis of their structure-carotenes (β-carotene, lycopene) containing only carbon and hydrogen that may be cyclic or linear and oxycarotenoids (xanthophylls, lutein) containing carbon, hydrogen and oxygen in the form of hydroxyl, epoxy or oxy groups *(Kaur et al., 2004; Rietjens et al., 2002)*. Carotenoids are polyene hydrocarbon compounds biosynthesized from eight isoprene units (tetraterpenes) and, correspondingly, have a 40-C skeleton. Figure 12 shows sructural formulae of

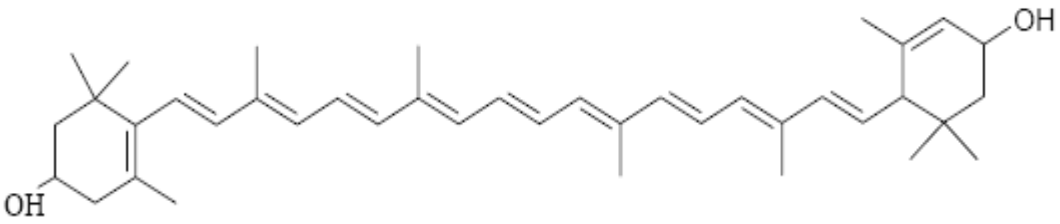
carotenoids (*Isler, 1971; Chichester, 1972; Zakaria et al.,1979; Bauernfeind, 1981; Belitz and Grosch, 1992).*



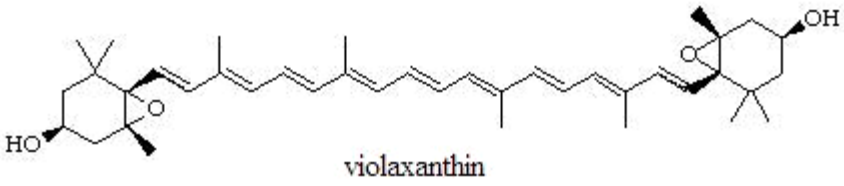
α-carotene



β-carotene



lutein



violaxanthin

Figure 12. Structural formulae of some carotenoids

Hydrogenation, dehydrogenation and/or cyclization of the basic structure of the 40-C carotenoids derive the other carotenoids. The latter reaction can occur at one or both end groups. Electrochemical oxidation of *all-trans*-canthaxanthin and β-carotene in dichloromethane leads to significant *trans*-to-*cis* isomerization,with *cis*isomers accounting for about 40% of the products formed.The electrochemically generated isomers were separated by reverse-phase high-performance liquid chromatography and identified as 9-*cis*, 13-*cis*, 15-*cis*, and 9,13-di-*cis* isomers of the carotenoids by ^{1}H-NMR spectroscopy and optical spectroscopy (*Q* ratio). The results of simultaneous bulk electrolysis and optical absorption spectroscopy indicate the following isomerization mechanism: the *all-trans* cation radicals and/or dications formed by electrochemical oxidation of *all-trans*-carotenoids can easily undergo geometrical isomerization to form *cis* cation radicals and/or dications. The latter are converted by the comproportionation equilibrium to cation radicals which are then transformed to neutral *cis*-carotenoids by exchanging one electron with neutral carotenoids. AM1 molecular orbital calculations, which show that the energy barriers of configurational transformation from *trans* to *cis* are much lower in the cation radical and dication species than in the neutral molecule, strongly support the first step of this mechanism (*Goa et al., 1996*) see (Figure 13, 14).

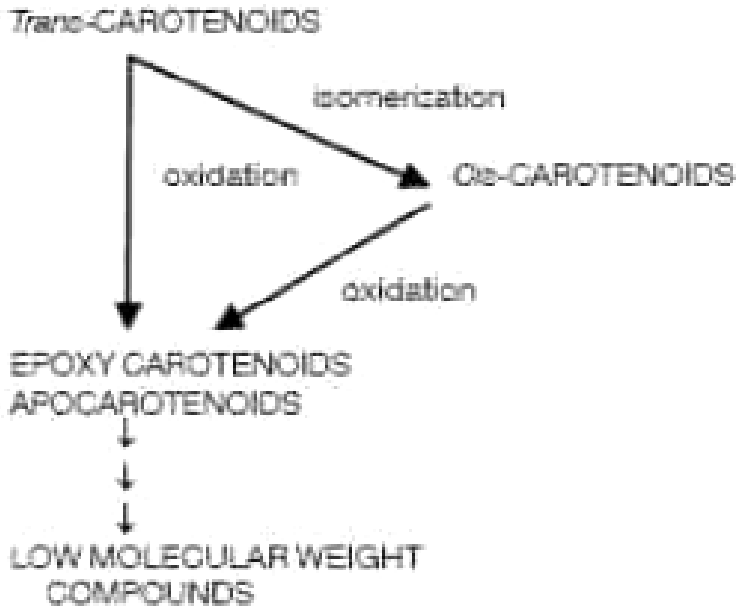
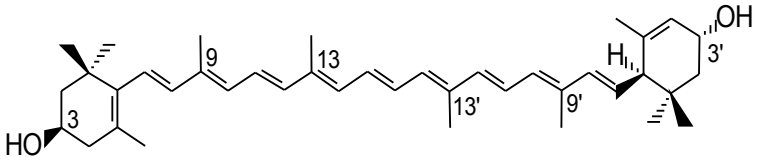
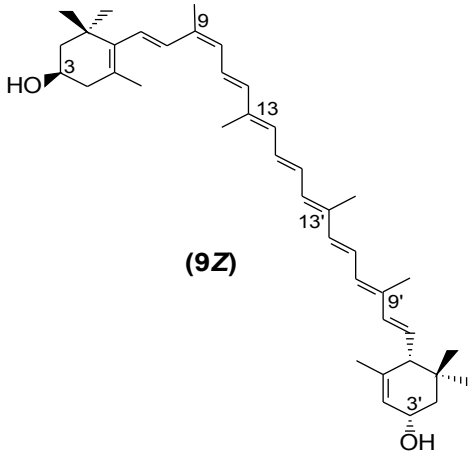


Figure 13. oxidation and isomerization of carotenoids



all-E



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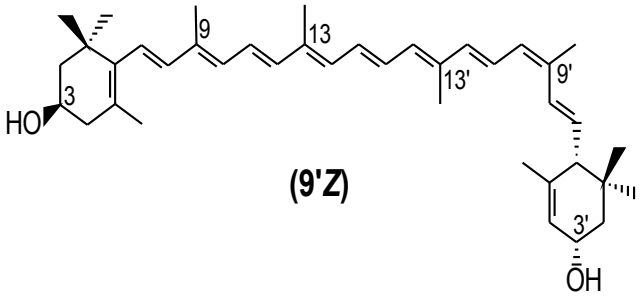
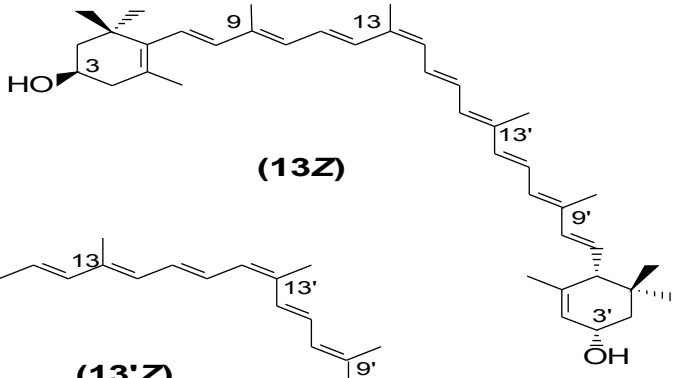
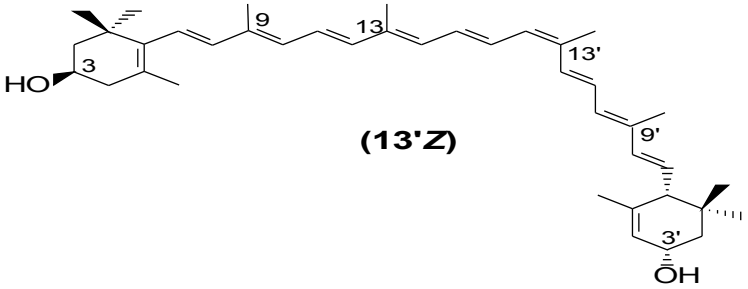


(9'Z)



(13Z)



(13'Z)

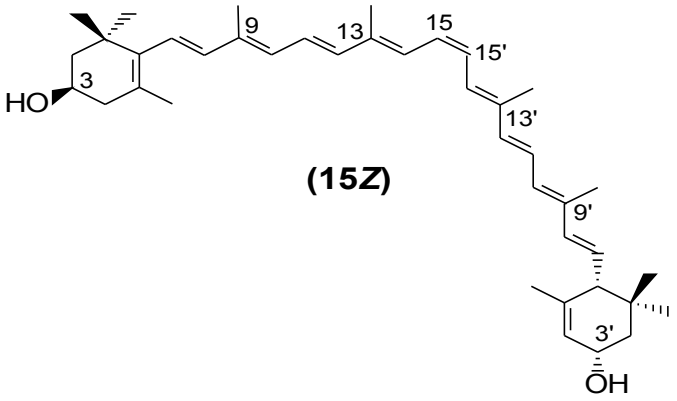


Figure 14. Carotenoid cis-isomers

It has long been demonstrated that two different pathways of the biosynthesis are distinguishable in higher plants; light dependent and light independent. In light-dependent pathways, carotenoids are formed together with chlorophyll in chloroplasts. The major carotenoids of such a pathway is are β-carotene, lutein, violaxanthin and neoxanthin. In some plants such as leafy vegetables, this results in a formation of considerable amounts of β-carotene that makes the products useful for the provision of dietary pro-vitamin A *(Khachik et al.; 1991).*

The light-independent pathway is initiated immediately after chlorophyll degradation as a function of ripening process in fruits and vegetables. Depending on the type, horticultural crops tomato and apricot contain mainly oxygen non-containing carotenes, whereas red pepper, citrus fruits and peach contain, in addition to carotene, fatty acid esters of OH-containing xanthophylls *(Biacs and Daood, 1994).*

It is well known fact that some dietary carotenoids, when absorbed, are converted to vitamin A in the intestine, a process which is under homeostatic control. β-carotene is transported by the chylomicrons to the liver *(Biesalski, 1994).* Non-provitamin A carotenoids such as lutein, lycopene, neoxanthin, violaxanthin have been found in blood and different organs of animal and human body revealing the high biological activity of such compounds.

Among the carotenes,only alpha, beta and epsilon carotenes possess vitamin A activity and out of these β-carotene is the precursor of vitamin A and has preventive action against eye diseases and cancer. Carotenes enhance immune response and protect skin cells against UV radiation. They help to lower the risk of cardiovascular diseases, age related vision disorders, asthma and reduce inflammation*(Kris-Etherton et al., 2002; Willcox et al., 2004).* On the other hand in vitro tests, β-carotene is an efficient scavenger of peroxyl radicals and its provitamin A activity is proved. Lycopene gives tomatoes their red color and is particularly effective at quenching the destructive singlet oxygen. Along with carotene

and lutein, it provides protection against lung, breast, uterus and prostate cancers. Green leafy vegetables and corn are best sources of xanthophylls like lutein and zeaxanthin that are believed to function as protective antioxidants for the retinal part of human eye. Astaxanthin, a xanthophyll found in salmon, shrimp and other seafoods with potent antioxidant properties *(George et al., 2004; Kaur et al., 2004; Stahl, 2005).* Limonoids, the second major subclass of terpenoids, are the biologically active phytochemicals present in citrus which act as antioxidants and protect lung tissues from free oxygen radicals. In vitro studies show that limonin, nomilin and limonoid glycosides have significant ability to inhibit proliferation of human breast cancer *(Ortuno et al., 2006).*

2.2.3.1.2 Chlorophylls

The green color in nature results from the presence of chlorophyll pigments that occur in the chloroplasts of plant tissues. The chloroplasts hold chlorophylls close to the cell wall and carry out photosynthesis on bio-membranes. In coordination with other pigments like carotenoids, chlorophylls play an important role in the metabolism of light energy and catalyze synthesis of carbohydrates, the key metabolites of the metabolism of living cells.

Because of their widespread occurrence in a variety of plant products, chlorophylls play a vital role in the acceptability of food commodities. Chlorophyll-containing products have the potential to be used as a natural food colorant and nutritional supplement. The changes in chlorophyll content and composition can be used as an indication for some physiological process and orders. Recently, biological studies have postulated the anticarcinogenic and antimutagenetic effect of chlorophyll-related compounds.

Chlorophyll is vital for photosynthesis, which allows plants to obtain energy from light. Chlorophyll is the molecule that absorbs sunlight and uses its energy to synthesize carbohydrates from CO_2 and water. This process is known as photosynthesis and is the basis for sustaining the life processes of all plants. Since animals and humans obtain their food supply by eating plants, photosynthesis can be said to be the source of our life also *(Stryer, 1975).*

$$6CO_2 + 6H_2O \xrightarrow[\text{chlorophyll}]{\text{Sunlight}} \underset{\text{(glucose)}}{C_6H_{12}O_6} + 6O_2$$

Green plants have five closely-related photosynthetic pigments (in order of increasing polarity):

Carotene - an orange pigment

Xanthophyll - a yellow pigment

Chlorophyll a - a blue-green pigment

Chlorophyll b - a yellow-green pigment

Phaeophytin – a grey pigment

Chlorophyll a is the most common of the five, present in every plant that performs photosynthesis. The reason that there are so many pigments is that each absorbs light more efficiently in a different part of the spectrum. Chlorophyll a absorbs well at a wavelength of about 400-450 nm and at 650-700 nm; chlorophyll b at 450-500 nm and at 600-650 nm. Xanthophyll absorbs well at 400-530 nm. However, none of the pigments absorbs well in the green-yellow region, which is responsible for the abundant green we see in nature *(Streitweiser and Heathcock, 1981).*

Chlorophyll is a chlorin pigment, which is structurally similar to and produced through the same metabolic pathway as other porphyrin pigments such as heme. At the center of the chlorin ring is a magnesium ion. The chlorin ring can have several different side chains, usually including a long phytol chain. There are a few different forms that occur naturally, but the most widely distributed form in terrestrial plants is chlorophyll *a*. The general structure of chlorophyll *a* was elucidated by Hans Fischer in 1940, and by 1960, when most of the stereochemistry of chlorophyll *a* was known, Woodward published a total synthesis of the molecule as then known.

The different structures of chlorophyll are summarized below:

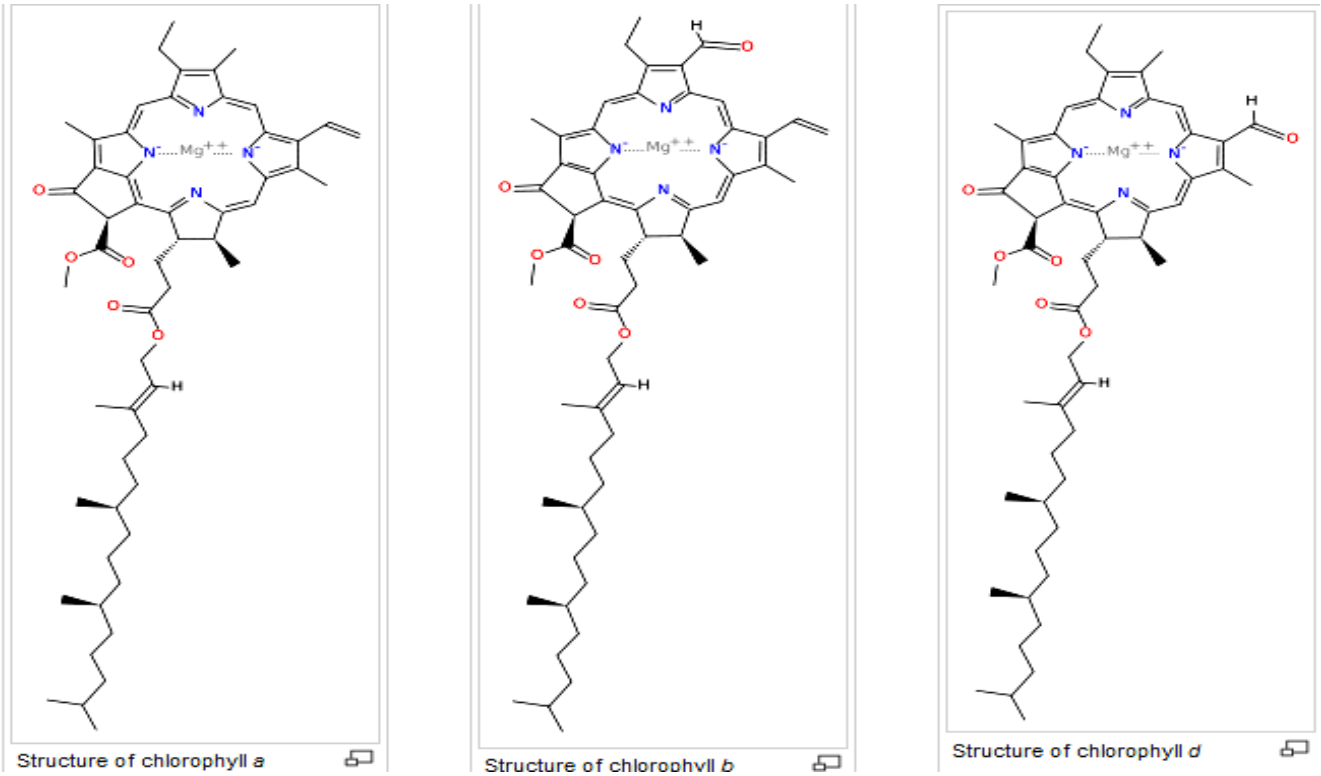


Figure 13. Different structures of chlorophll

Chlorophyll *a* occurs in universal,chlorophyll *b* occurs mostly in plants, chlorophyll *c* occurs in various algae, where chlorophyll *d* occurs in cyanobacteria.

Degradation of chlorophyll usually happens all the way from ripeness to processing and storage of plant-derived foods.The mechanism of degradation still remains fragmentary. Nevertheless it is generally assumed that chlorophyll is degraded to colorless products, thus exposing the carotenoids. The results of several investigations suggest that chlorophyll is first degraded to the phytyl-free chlorophyllides by the enzyme chlorophyllase, followed by oxidative degradation by peroxidase, lipoxygenase,or chlorophyll oxidase. The simultaneous action of dechelase may raise the diversity of chlorophyll extract by producing the corresponding Mg-free derivatives of the aforementioned chlorophyll degradation products as shown in (Figure 16). It should be noted that further degradation of chlorophylls and their derivatives to colorless compounds of low molecular weight is a function of oxidation processes which is not yet well described.

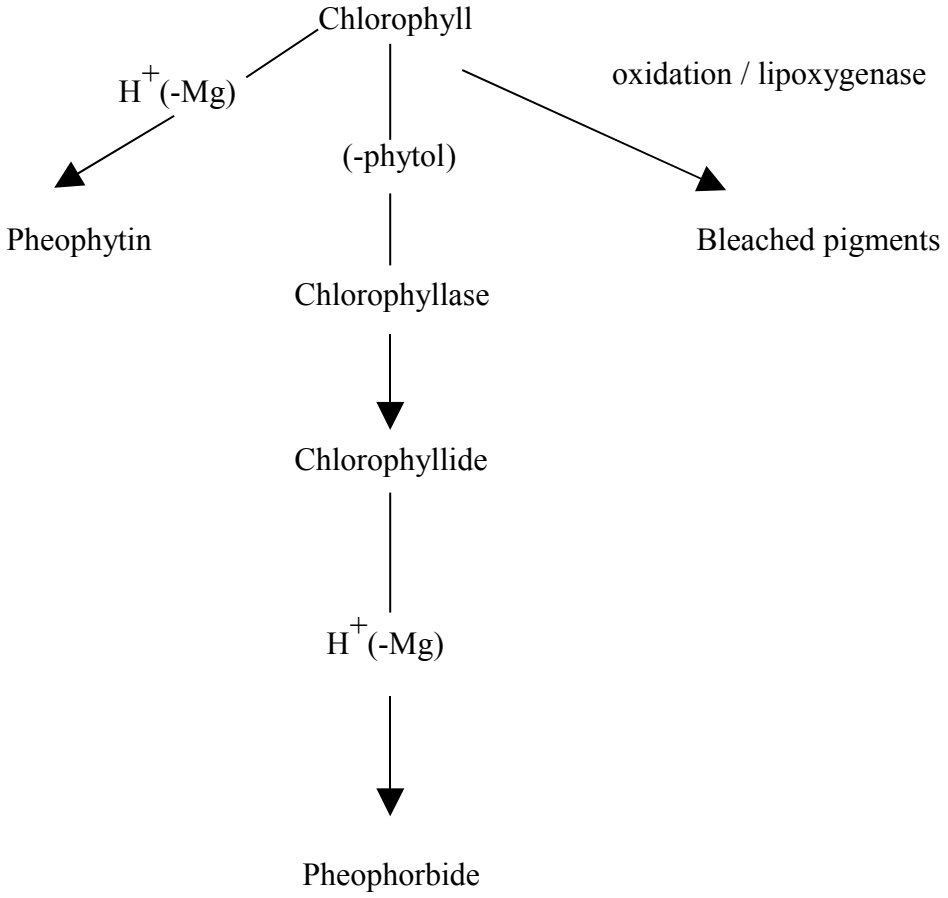


Figure 16 . Degradation of chlorophyll

Due to their chemical nature and hydrophobic properties, chlorophylls are able to interfere with the chain of lipid oxidation in food. From industrial practices and *in vitro* experiments, chlorophylls and their degraded products can play the role of either antioxidants or prooxidants. Factors which are most likely to determine the antioxidative or oxidative activity of chlorophylls include variety, ripeness stage, and chemical composition of food where they exist, the presence or absence of effective oxidants or antioxidants, and the climate. When tested by ferric thiocyanate and other assays, chlorophylls and their derivatives exhibit marked antioxidant activity. Recent studies show that acidic fractions from plant containing pheophytins and related compounds have antioxidant effect which is higher than that of α-tocopherol and comparable to that of BHT (butylated hydroxytoluene). The antioxidant activity of chlorophyll-derived compounds is attributable to their interference with free radical cycles as scavengers to neutralize highly reactive oxygen-free forms such as superoxides. The porphyrin ring system of chlorophylls seems to be important for the antioxidant activity.

Treating some foods with oil containing chlorophyll significantly reduces peroxide value during storage in the dark. This gives convincing evidence on the antioxidant effect of chlorophylls. In dry, oiled, and toasted laver, for example, the high lipid oxidative stability is ascribed to the presence of chlorophylls in the oil used. A positive correlation between oil stability and chlorophyll content is recognized in the processing of virgin oil.

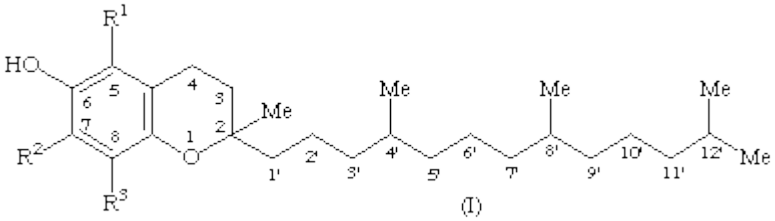
2.2.3.1.3 Tocopherols

Vitamin E is a family of compounds known as tocopherols and tocotrienols, of which α-tocopherol is the major component and the most biologically active form. These redox-type lipids are of nutritional/physiological and analytical interest. Vitamin E is fat-soluble; it is the major antioxidant vitamin transported in the bloodstream by the lipid phase of the lipoprotein particles. Vitamin E is found in nature, primarily in cereals (especially wheat germ oil), nuts and vegetable oils *(Esterbauer, 1991).*

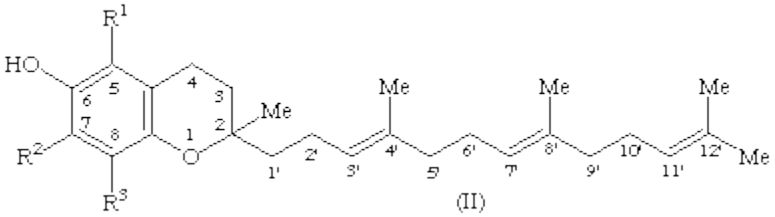
Tocopherols and tocotrienols are non-polar constituents of biological membranes that exist in nature in lipid phase. Tocopherols consist of a chromane ring and a long saturated phytyl chain. Tocopherols commonly known as tocols are 2-methyl-2-(4', 8', 12'-trimethyl tridecyl) chroman-6-ols. When 3 double bonds are present at positions 3', 7' and 11' of the side chain in tocols they are called tocotrienols. The α-, β-, γ-, and δ- tocols and tocotrienols differ in the number and position of methyl groups attached to the 5, 7 and 8 position of the ring structure Figure 17. α-tocopherol is the most abundant form, with high vitamin E activity and singlet oxygen quenching ability than other forms of tocopherols but is less effective in scavenging superoxide anion generated by xanthine oxidase *(Papas,*

1999; Rietjens et al., 2002). γ –tocopherols can reduce the concentration of nitrogen dioxide most effectively that is involved in carcinogenesis, arthritis and neurologic diseases. Efficiency of scavenging hydroxyl, alkoxyl and peroxyl radicals by α- tocopherols is approximately 10^{10}, 10^{8}, and 10^{6} (M/s) respectively *(Larson, 1997; Kornsteiner et al., 2006).* Antioxidant mechanisms of tocopherols mainly involve the transfer of hydrogen at 6-hydroxyl group of a chromane ring, scavenging of free radicals and regeneration in the presence of ascorbic acid. Their phytyl chain adjusts itself in membrane bilayer while active chromane ring is closely positioned to the surface. This unique structure enables them to act as effective antioxidants and to be regenerated through reaction with other antioxidants *(Gliszczynska-Świglo, 2006; Packer and Weber, 2001*). The antioxidant activity decreases from delta through alpha, while vitamin E activity increases in the reverse series. *Dugan, (1980)* indicated that tocopherols have been found to be effective as antioxidants in a number of products, including bacon, baked goods butterfat, lard, margarine, rape seed oils, safflower oil, and sunflowers oil. Tocopherols show their greatest potency as antioxidants in animal fats, carotenoids, and vitamin A *(Cort, 1974).*Tocotrienols mainly found in palm oil, cereal grains and kale are also potential antioxidant and their mechanism of action is similar to tocopherols. They are associated with the reduced risk of cancer, Alzheimer's and cardiovascular diseases, cholesterol lowering ability and inhibited LDL oxidation *(Park and Pezzuto, 2002)* α –tocotrienol is preferentially absorbed as compared to its δ- and γ- form. Even though tocotrienols have a higher radical scavenging activity than tocopherols but they are less bioavailable as compared to the latter (*Bast and Haenen, 2002).*

Different structures of chlorophyll are summarized below in (Figure 17).



	R1	R2	R3
α-Tocopherol	CH3	CH3	CH3
β- Tocopherol	CH3	H	CH3
γ-Tocopherol	H	CH3	CH3
δ-Tocopherol	H	H	CH3



Tocotrienols

	R1	R2	R3
α-Tocotrienol	CH3	CH3	CH3
β- Tocotrienol	CH3	H	CH3
γ-Tocotrienol	H	CH3	CH3
δ-Tocotrienol	H	H	CH3

Figure 17. Tocopherols and Tocotrienols

2.2.3.1.4 Essential oils

They are complex and highly variable mixtures of constituents which belong, virtually exclusively to two groups characterised by distinct biogenetic origins: the group of terpenoids, and the group of aromatic compounds derived from phenylpropane. Some essential oils contain degradation products of non-volatile constituents. Terpenoids are mainly volatile terpenes (with light molecule weight): mono (C10Hx)- and sesquiterpens (C15). Among terpenoids we can found acyclic and cyclic mono- and sesquiterpens with different multiple functionalized molecules as followings: alcohols (e.g., linalool, terpinen-4-ol, borneol), aldehydes (e.g., geranial, neral), ketones (e.g., carvone, menthone, camphor), esters (e.g., linalyl acetate, terpenyl acetate), peroxides (e.g., ascaridol), phenols (e.g., thymol, carvacrol). Phenylpropanoids (C_6-C_3) are very often allyl- and propenylphenols and sometimes they are aldehydes (anethole, methylchavicol, eugenol). Essential oils contain compounds arising from fatty acid degredation (odour, flavour compounds: such as octanal, hexanal, decanal) and compounds arising from terpene degradation (arise from the autoxidation of carotenes, e.g., damascones).

The essential oils isolated from plants, flowers, seeds and spices have been investigated for decades mainly due to their significant applications in perfumery, flavoring agents, paints and other industries (*Aburjai and Natsheh, 2003; Pybus et al., 1999; Hay and Waterman, 1993*).

Cardamom contains a bioactive essential oils. The main essential oils found in cardamom are (1,8 cineol, linalool, terpin-4-ol, α-terpineol, linalyl acetate, and α-terpinyl acetate) (*Lucchesi et al., 2007*). Essential oil of cardamom is the source of its aroma and flavour, strongly tonic and stimulant, stomachic and carminative (*Franchomme and Péoél, 1990*).

Green leaves of palestinian thyme are rich in essential oil, which is responsible for its characteristic flavor and fragrance (*Baser et al., 1993*). The genus *Thymus* includes about 350 species word-wide. The chemical composition of the essential oils of *Thymus* species have been extensively studied due to their biological importance (*Stahl-Biskup, 1991).*Mainly essential oils of citrus fruits, cherries, mint and herbs has been reported as antioxidant agent *(Mantle et al., 1998).* Plants belonging to the Lamiaceae family are known to be rich in compounds possessing strong antioxidant activity (*Youdim et al., 1999: Miura et al*., 2002).The essential oils of the genus *thymus* are mainly monoterpenic (C_{10}), and often contain large amount of thymol and carvacrol which inhibit the peroxidation of liposome phospholipids in a concentration-dependent manner (*Aeschbach et al., 1990*).

Kulisic studied the antioxidant properties of thyme and wild thyme essential oils. He found that the essential oils found in *Thymus vulgaris* and *Thymus serpyllum* (γ-Terpinene, ρ-Cymene, Thymol, and

Carvacrol) have an antioxidant activity (thyme is slightly better than wild thyme) (*Kulisic et al., 2005*). Also (*Kulisic et al., 2004*) studied showed that oregano essential oil that contain a high content of thymol and carvacrol has strong antioxidant activity. Essential oils of thyme, oregano, and basil studied by Biljana research team, expressed a strong antioxidant activity (*Bozin et al., 2006*).

Monoterpens have shown efficacy in both cancer prevention and therapy, they have been reported to decrease the incidence of chemically induced tumors in skin, liver, lung, breast and fore stomach of rats (*(Kris-Etherton et al., 2002*).

2.2.4. Antimicrobial properties of medicinal herbs and extracts

Medicinal plants, herbs, spices and their different extracts have been used against microbes (bacteria, fungi) since thousand years. In folklore usage these herbs and extracts have been applied for this purpose without having knowledge about the exact effects and side-effects of the herb or its extract. To understand of which part of plant is useful, which compounds are responsible for the antimicrobial effects and how these certain compounds work in synergist or individual way have been studied recently as these molecules can be base of synthetic pharmaceuticals or can be applied as extracted directly from the natural sources. One of the driving factors for the renewed interest in plant antimicrobials has been the rapid rate of species extinction. Also the costumers require more high quality, preservative-free or naturally preserved, safe but mildly processed foods with extended shelf-life. Most traditional food preservation processes have been developed empirically without a full understanding of the mechanisms of action of the antimicrobial agents used (*Cowan, 1999; Kilsby*, *1999*).

2.2.4.1. *Antimicrobial compounds from plants*

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivates. Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavour and some of the same herbs and spices used by human to season food yield useful medicinal compounds. These antimicrobial phytochemicals can be divided into several categories, described below.

2.2.4.1.1. *Phenolics and Polyphenols*

***Phenolic acids*.** Caffeic acid from tarragon and thyme has been reported to be effective against bacteria, fungi and viruses (*Bernáth, 2000; Cowan, 1999*). Cinnamic acid, vanillic acid and ferulic acid have been found to have anti-*Listeria monocytogenes* activity *(Brul and Coote, 1999*).

***Coumarins*.** Coumarins are known to be highly toxic in rodents. Warfarin is a well-known coumarin, which is used both as oral anticoagulant and as rodenticide. Coumarin was found in vitro to inhibit *Candida albicans*. As a group, coumarins have been found to stimulate macrophages, which could have an indirect negative effect on infections (*Cowan, 1999*).

***Flavones, flavonoids and flavonols*.** They have been found numerously in vitro to be effective antimicrobial substances against a wide array of microorganisms. Lipophilic flavonoids may disrupt microbial membrane. Flavonoid compounds exhibit inhibitory effects against multiple viruses. It has been reported such as swertifrancheside and chrysin against HIV. Kaul et al. provide a summary of the activities and modes of action of quercitin, naringin, and hesperetin in in vitro cell culture monolayers. The molecules, except naringin were inhibitory to herpes simplex virus type I (*Kaul, 1985*). An isoflavone found in a West African legume, alpinumisoflavone prevents schistosomal infection. Galangin found in *Helichrysum aureonitens* seems to be a useful compound against gram-positive bacteria, fungi and viruses (*Cowan, 1999; Kilsby, 1999; Kaul et al., 1985)*.

Tannins. Catechins have been widely studied due to their occurrence in oolong green teas. The tea exerted antimicrobial activity. These compounds inhibited in vitro *Vibrio cholerae*, *Streptococcus* mutans*, Shigella*, and other bacteria. When rats were fed a diet containing 0.1% tea catechins, fissure caries (caused by S.mutans) was reduced by 40%. Tannin-containing beverages (green teas, red wines) can cure and prevent a variety of illness. Tannins in plants inhibit insect growth and disrupt digestive events in ruminal animals (*Bernáth, 2000; Cowan, 1999*).

***Quinones*.** They arise from oxidation of phenols, have aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive (e.g., naphtoquinone, anthraquinone). Kazm et al. described an anthraquinone from *Cassia italica* which was bacteriostatic for *Bacillus anthracis*, *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae* (*Kazm et al., 1994*). Hypericin, a naphthodianthrone from Hypericum perforatum has antimicrobial properties although its antidepressant effect is better known (*Bernáth, 2000; Cowan, 1999; Kazmi et al.,1994*).

2.2.4.1.2. Terpenoids

Essential oils. Terpenes and terpenoids are the mostly noted agents against bacteria, fungi, viruses and protozoa. Among them the most active are: thymol, eugenol, carvacrol, etc (*Bernáth, 2000; Brul and Coote, 1999; Cowan, 1999; Adams and Moss, 1994*) Certain examples are summarized within the chapters describing the thyme, cardamom and cardamom parika mixture examined in this research work.

Diterpens. They constitute a vastgroup of C_{20}compounds arising from the metabolism of geranylgeranyl pyrophosphate.They exist in acyclic and cyclic (bi-, tri- and tetracyclic) forms. Rosmanol and carnosol the highly interesting molecules belong to diterpens. Kaurene diterpenoid was reported against microba (*Bernáth, 2000; Cowan, 1999*).

Saponins. They are characterised by their surface-active properties: they dissolve in water to form foamy solutions. Structurally, saponins may be classified into two groups based on the nature of their aglycone. These are: steroidal saponins (e.g., spirostane, digitogenin, etc.) and triterpenoid saponins (e.g.,oleanolic acid, hederagenin, etc.). Among triterpenoid saponins, betulinic acid and glycyrrhizin (from licorice) have been shown to inhibit HIV (*Bernáth, 2000; Cowan, 1999*).

2.2.4.1.3. *Alkaloids*

Heterocyclic nitrogen compounds are called alkaloids. The first medicinally useful example of an alkaloid was morphine isolated in 1805 from the opium poppy. Codein and heroin are both derivates of morphine. A protoalkaloid, capsaicin from *Capsiucum annuum* showed although mixed, but antimicrobial activity. Recently, Cichewicz and Thorpe found that capsaicin might enhance the growth of *Candida albicans* but it clearly inhibited various bacteria. Although possibly detrimental to the human gastric mucosa, capsaicin is also bactericidal to *Helicobacter pylori* (*Cichewicz and Thorpe, 1996*). Solamargine, a steroidal glycoalkaloid from the berries of *Solanum khasianum* may be useful against HIV infection as well as intestinal infections associated with AIDS. Berberine (tertiary tetracyclic alkaloid) is potencially effective against *trypanosomes* and *plasmodia*. The effects of these highly aromatic planar quaternary alkaloids, such as berberine and harmane are attributed to their ability to intercalate with DNA (*Bernáth, 2000; Cowan, 1999*; *Cichewicz and Thorpe, 1996).*

2.2.4.1.4. *Other compounds*

Peptides which are inhibitory to microorganisms were first reported in 1942. Recently interest has been focused mostly on studying anti-HIV peptides and proteins. Thionins are peptides in barley and wheat and toxic to yeasts and gram-negative and gram-positive bacteria. Another peptide isolated from fava beans, fabatin inhibits *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* but not *Candida* or *Saccharomyces*. Larger lectin molecules (example: jacalin) inhibit viral proliferation (HIV) (*Cowan, 1999*). Other compounds like amino acid derivates from garlic and onion, namely isothiocyanates possess strong antimicrobial properties. Considerable amounts of allylisothiocyanates and methylisothiocyanates have been used in the agricultural industry as pesticides, while their direct application in foods is hampered by the intense sensory attributes of these compounds (*Brul and Coote, 1999*).

2.2.4.1.5. *Mixtures*

Generally the essential oils, solvent extracts or other crude extracts of herbs have been used in antimicrobial tests, therefore the observed inhibitory effects might be the final result of the antimicrobial effects of all compounds and/or synergist effects among the compounds. High numbers of publications can be found on antifungal and antibacterial behaviour of certain herb extracts. It can be concluded that the *Lamiaceae* herbs might possess stronger antimicrobial properties, although herbs belong to *Boraginaceae*, *Apiaceae*, *Rubiaceae* and *Plantaginaceae* plant families were reported as antimicrobial agents (*Cowan, 1999*).

It is very important to understand the effects of each biological active compound although I believe that the herb or its extracts might have better, stronger and more valuable properties containing all the biological active compounds with the synergist possibility.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 *Raw materials*

40 kilograms of fresh thyme (*Thymus serpyllum*) were obtained from Palestine in 2004. The batch was naturally dried (2-3 days sun dry), the water content was (8-10%), and ground by coffee mill (to pass 20 mesh sieve). The ground thyme was packaged in a nylon sack with slight vacuum and transferred to the laboratories of the Central Food Research Institute by airplane.

Half (0.5) kilogram of fresh thyme and about 250 gram of ground sample were stored at -20 C° for the chemical analysis of the starting materials.

High qualities of unknown variety Indian cardamom seeds were obtained from the local market of Syria, Damascus. Before extraction, the seeds were grinded by coffee mill (to pass 20 mesh sieve) for the extraction experiment. Part of the purchased quality was stored at -20 C° in a vacuumed nylon sack for chemical analysis of starting materials.

3.1.2 *Chemicals used*

Analytical grade organic solvents such as methanol, ethyl acetate, acetone, 1, 2 dichloroethane, hexane, petroleum ether, potassium dihydrogen phosphate, sodium sulphate (anhydrous), phosphoric acid, and potassium hydroxide were obtained from Reanal (Budapest, Hungary). HPLC grade acetonitrile, isopropanol, methanol, absolute ethanol and hexane were purchased from Merck (Darmstadt, Germany). Standard materials such as chlorophyll A and B, β-carotene, tocopherols, as well as tetrabutyl ammonium hydroxide were purchased from Sigma (St. Lo. USA). Technical grade of CO2 and propane 99.9% purity were purchased from Messer Griesheim Hungaria (Budapest, Hungary).

3.2 Chemical analyses

3.2.1 *Analysis of carotenoids and chlorophylls*

3.2.1.1 *Extraction of carotenoids and chlorophylls*

To analyse the chlorophylls and carotenoids in ground thyme leaves and in cardamom seeds, 0.5-1 g of ground thyme and 2 g of cardamom seeds were extracted twice by mechanical shaking with 50 ml of 2:1:1 dichloroethane-acetone-methanol for 15 minutes. The mixtures were then filtered through a filter paper, and the solvent evaporated under vacuum at 35-40 ˚C using rotary evaporator.

In case of oleoresin extract of thyme and cardamom, 50-100 mg of each were dissolved in 5-10 ml of the HPLC eluent consisting of 10:35:55 methanol-acetonitrile-isopropanol with the help of a water bath

ultrasonic device (Tesla, Czech Republic) for 30 seconds followed by filtration through a 45 μm Teflon syringe filter before injection into the HPLC column.

3.2.1.2 ***Conditions of HPLC analysis***

Instrument: The high-performance liquid chromatographic (HPLC) analysis were carried out using a Waters (Milford, MA, USA) Alliance chromatograph consisting a Model 2695 separation module and a Model 2996 photodiode-array detector. The system was operated and the data processed by Empower software.

Column: The chlorophylls and carotenoids were separated on Nucleosil 100, 5μm, C-18, 250x46 mm, end capped column (Macherey-Nagel, Düren, Germany).

Mobile phase*:* The mobile phase consisted of (A) 10% water in methanol and (B) 35:55:10 acetonitrile-isopropanol-methanol with gradient elution starting with 100% A, changed in 20 minutes to 100% B, which kept isocratic for further 5 minutes and then turned to 100% A in 5 minutes (*Daood and Biacs 2005*).

Flow rate: The flow rate was 0.9 ml/min.

Detection: The effluents were monitored at 450 and 655 nm for the quantification of carotenoids and chlorophylls respectively.

Identification: Standard β-caroten, lutein, chlorophyll a and b (Sigma-Aldrich) were used for their identification and quantification. In the qualitative analysis, the scanned spectra and retention times of the individual compounds were compared with those of standard and authentic materials and with literature data as well (*Baurenfiend, 1981*). To tentatively identify the oxidised chlorophyll derivatives, standard chlorophyll a and b were subjected to a lipoxygenase-catalysed linoleic acid co-oxidation reaction in a previously described experimental system (*Daood and Biacs, 1988*). Mg-free pheophytins were identified by comparing retention times and spectral characteristics with those of authentic materials prepared by acidification of acetone solutions of standard chlorophylls a and b with diluted HCL solution (0.01M).

3.2.2 Analysis of tocopherols

3.2.2.1 ***Extraction of tocopherols***

To analyse tocopherols, 1g of raw material or 100 mg of the extract were saponified with methanolic KOH for 35 min at the boiling point of methanol in presence of 0.5 g ascorbic acid. After cooling and addition of salted water the tocopherol fraction was extracted twice by gentle shaking with 40 ml of n-hexane. The hexane layers were separated and pooled, then washed three times with distilled water and dried over anhydrous Na_2SO_4. After vacuum removal of solvent, the dry residues were collected in 10 ml of HPLC-grade n-hexane.

3.2.2.2 *Conditions of HPLC analysis*

Instrument: Waters (Milford, MA, USA) Alliance chromatograph (HPLC) series consisting of a Model 2695 separation module and a Model 2475 fluorescent detector.

Column: Tocopherol fraction was separated into different analogues on normal phase Nucleosil 100, 10 μm, 240 x 4.6 mm column.

Mobile phase: The mobile phase was (99.6:0.4) n-hexane-absolute alcohol *(Speek . et al., 1985).*

Flow rate: The flow rate was 1.2ml.

Detection: The compounds were detected by fluorescence detector at Ex: 295nm and Em: 330 nm. The detector signals were electronically recorded and integrated.

Identification

The peaks of HPLC chromatogram of tocopherol fraction were identefied by comparing their retention times and spectral characteristics with those of standard tocopherols prepared at suitable concentrations.

3.2.3 Validation

Recovery test: From stock solutions of 0.1mg/ml 10 –20 μg of the standard materials were added (in triplicate) to 2 g of the food samples with mixing and homogenization in a crucible mortar. The samples were stored in dark place for the solvents to evaporate and then subjected to the analytical procedures. The % recovery was calculated by the difference between non-spiked spiked and spiked in the quantities of the analytes and relating the found to the added quantities.

Precision test: Precision in terms of within day repeatability, expressed as relative standard deviation (% RSD) was calculated from the values obtained from five measurements including extractions and HPLC analyses of a well- homogenized thyme and cardamom seeds

Limit of Detection and Quantification (LOD, LOQ): The limits of detection and quantification were measured by calculating the concentration of the analytes in μg/ ml at a ratio of peak/noise of 3 and 10 respectively. With Waters Alliance chromatograph the PDA or absorbance detector start with showing on screen the maximum noise assisting in rapid estimation of these values. In case of integrator it was needed to raise the sensitivity (Attenuation) to the maximum and to inject the analytes at very low concentration.

Calibration: Linearity of calibration curves for the different standard materials was tested by plotting the integrated area of the peaks on the HPLC chromatograms versus different concentration starting from a concentration higher than the LOQ. The range of concentrations used for the different compounds varied from compound to another as shown in results and discussion.

3.2.4 Analysis of fatty acids

3.2.4.1 *Extraction*

After weighting and diluting with a known amount of chloroform, an aliquot of the sample containing 7 mg of fresh oil was transferred to a screw capped vial and the solvent evaporated under an N_2 stream. After saponification with 0. 5 mol 1^{-1} KOH / methyl alcohol, the sample was esterified with $BF_{3/}$ methyl alcohol. The esters were salted out with saturated NaCl solution into n-heptane dried on sodium sulfate *(Hungarian Standard, 1979),* 0. 3µl was used for chromatography.

3.2.4.2 *GC conditions*

Chromatographic separation was performed on Hewlett-Packard 5720A chromatograph equipped with FID and HP 3392A type integrator. The chromatographic conditions found best for fresh oils were as follows:

Column*:* 0. 75 mm i.d., 30 m long, wide bore borosilicate capillary column with 1µm thick Supelcowax.

Carrier gas: hydrogen, linear velocity 30 cm sec^{-1}.

Temperature program: 180 °C -4°C- up to220 °C final temperature.

***Identification*:**

For the identification of fatty acid methyl esters standard (FAMEs) and standard oil mixtures were used.

3.2.5 Analysis of aroma compounds by GC-MS

3.2.5.1 *Qualitative analysis*

Aroma compounds of oleoresins from cardamom seeds alone or mixed with paprika seeds were extracted by solid phase micro extraction (SPME). Approximately 200 µl from each sample were put in 40 ml screw cap vial with septum and let to equilibrate at room temperature for 1 hour. The apolar polydimethyl-siloxan (PDMS) was used as the adsorbent in the SPME. In order to remove the contaminants coming from the laboratory air, PDMS fibre was heated for 10 minutes at injector temperature before inserting into sample vial. The PDMS fiber was kept for 30 minutes in the headspace over the sample. The desorption was performed in the injector of GC-MS instrument for 1 minute, meanwhile the split was kept closed.

3.2.5.2 *Quantitative analysis*

In the case of quantitative determination the samples were prepared by a different method with the use of 3-metyl-butyl- n-pentanoate as the internal standard (IS). 100 µl of oleoresins were weighted in a 10 ml volumetric flask and 2 ml of the IS stock solution was added which contained 25,1 mg 3-methyl-

butyl-n-pentanoate. The volume was adjusted to 10 ml by diethyl ether. From this solution 2 µl were directly injected into the GC-MS instrument. In the analysis of volatiles from the raw materials (ground thyme and cardamom seed) 400 mg was exactly weighted and 200 µl internal stock solution was added. After half hour shaking and settling, the clear supernatant was injected onto the capillary column of GC. For qualitative and quantitative analysis, gas chromatography-mass spectrometry was applied under the following conditions:

3.2.5.3. GC *conditions*:

Instrument: The separation and identification of volatile components was carried out by gas chromatography-mass spectroscopy (GC-MS). A HP 5890 gas chromatograph coupled with a HP 5971 quadrupol mass selective detector was used for this purpose.

Column: The column was RH-5ms 30 m x 0.25 mm i.d., 0.25 µm film thickness.

Carrier gas: Helium (purity 4.8) was used as a carrier gas.

Temperature program: 60 °C for 10 minutes, increased to 200 °C at 10 °C/ min, and 5 minutes at final temperature.

Injection: 260 °C.

Direct injection: split mode and 3 min solvent delay,

SPME: splitless for 1 min and no solvent delay.

Identification: Using Wiley 275 library data.

***3.3* Super and sub-critical extraction**

3.3.1 Extraction of thyme

Forty five grams of dried wild thyme were put in the extractor. (For volatiles and antimicrobial tests 700 g of dried thyme were put in the extractor of another supercritical extraction system found at Technical University of Budapest). SF extractions were performed with a high-pressure, flow-up stream extraction apparatus, shown in (Figure 18) .A membrane pump (Model EL-1 from Lewa Herbertott, Leonberg, Germany), pumped the solvent to the extractor at a flow rate of 1.0-1.5 l/min. The solvent passes through a buffer vessel into the thermostat-controlled column. A back-pressure valve regulator adjusted the pressure in the extractor. The solute-rich compressed gas was expanded to atmospheric pressure through a heated needle valve. The extracts were received in a cool container and left to stand for 20 min to warm allowing the solvent to evaporate. For various time intervals, the stable weight of the extracts was measured. The mass of solvent used was determined by applying Peng-Robinson equation *(Peng and Robinson, 1976)* after the normalization of the volume.

$$\left(P + \frac{a(T)}{V(V+b) + b(V-b)}\right)(V-b) = RT$$

Where:

P : pressure, v: molar valume, R: gas constant, T: temperature, a: attraction parameter, b: van der Waals co-volume.

The density of SC-CO_2 was calculated by applying Bender equation *(Bender, 1970)*.

$$P = R \,.\, T.\, \rho + B.\, \rho^2 + C.\, \rho^3 + D.\, \rho^4 + E.\, \rho^5 + F.\, \rho^6 + (G + H.\, \rho^2).\, \rho^3 \,.\, \exp(-a_{20} \,.\, \rho^2)$$

Where:

$$B = a_1.\, T - a_2 - a_3 / T - a_4 / T^2 - a_5 / T^3$$

$$C = a_6.\, T + a_7 + a_8 / T$$

$$D = a_9.\, T + a_{10}$$

$$E = a_{11}.T + a_{12}$$

$$F = a_{13}$$

$$G = a_{14} / T^2 + a_{15} / T^3 + a_{16} / T^4$$

$$H = a_{17} / T^2 + a_{18} / T^3 + a_{19} / T^4$$

And: $a_1, a_2, a_3, \ldots\ldots\ldots\ldots, a_{20}$ are constants.

Where: P : pressure. R: gas constant, ρ: density.

SFE studies were conducted to extract ground thyme leaves with SC-CO_2 at 35-55°C and pressure of 100-400 bar. In the case of sub-critical propane, the extraction was carried out at 25°C and pressure of 30-50 bar.

3.3.2 Extraction of cardamom

In case of cardamom 45 grams of freshly ground seeds, were dried (steam dry at 100-105°C in an oven for one night, water content was 11,7% and coffee-mill ground > 600μm) and packed in the extractor which have been previously described (*Illés et al., 1997*). SFE studies were conducted to extract ground cardamom seeds with SF-CO_2 at 25°C-55°C and pressure of 80-450 bar. In the case of sub-critical propane, the extraction was carried out at 25°C and pressure of 20-50 bar. Mixture of CO_2 and

modifier was prepared by mixing ethanol at a ratio of 25% (w/w) with liquid CO_2 in a cylinder one day before use. The cylinder was turned upside-down to ensure homogenity of the mixture. Ground paprika seeds alone and mixed 1:1 with ground cardamom seeds were also extracted by the same method at pressure of 300-450 bar and 40°C.

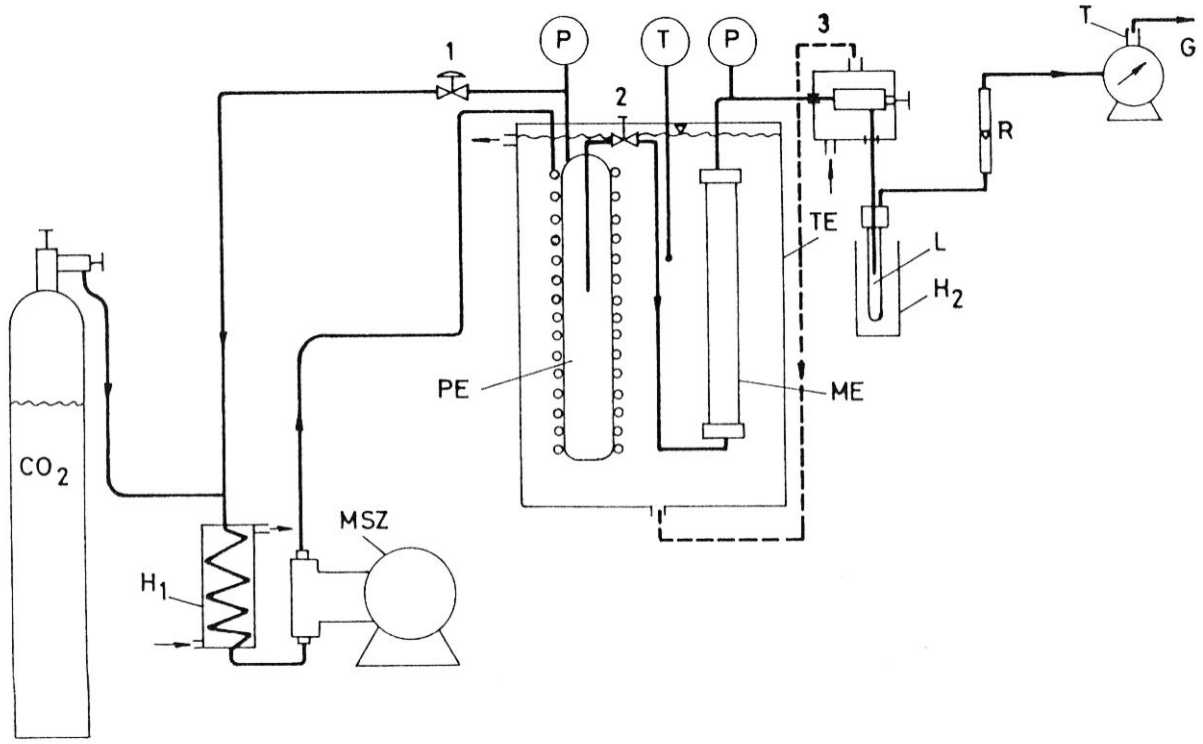


Figure 18. Supercritical fluid extraction system

G, gas-meter; H, cooler; L, cooled vessel; Me, extraction column; Msz, Membrane pump; PE, buffer vessel; R, rotameter; TE, thermostat; P, pressure-gauge; T, thermometer; 1 and 2, valves; 3, heated valve.

3.3.3 Organic solvent extraction

As a control in this work, 5 g of cardamom seeds and 0,5 g of thyme were extracted twice by shaking for 1 h with 200 ml of 2:1:1 1,2-dichloroethan-acetone-methanol for the determination of carotenoids and chlorophylls in the starting materials (ground thyme and cardamom seeds). The mixture was then filtered and the solvent evaporated under vacuum using rotary evaporator at 30°C. The recovered oil was transferred to small vials and kept under N_2 gas at refrigeration temperature. In the case of tocopherols and volatiles soxhlet extraction using petroleum ether 40˚-60˚ was carried out as the control.

3.4. Statistical Analysis and Calculation

Values are means ± SD. Data were analyzed using Statistical System software (Microsoft Office Excel 2000). Outcomes of interest (i.e. yield of oleoresin, concentration of carotenoids, chlorophylls, volatile oils, tocopherols, fatty acids in the extracts) among samples were analyzed by using one-way ANOVA at $P< 0.05$. Differences between treatment groups (extraction pressure and temperature and modifiers) were determined using Least Significant Differences at $P< 0.05$.

3.5. Determination of antioxidant capacity

3.5.1. Measurement by DPPH method in methyl alcohol

A spectrophotometric analysis that used DPPH was performed. It is based on the ability of the antioxidant to scavenge the radical cation DPPH. Data were expressed as trolox equivalent antioxidant activity (TEAC) using a Trolox calibration curve.

Sample preparation

The dry raw materials were grinded in a coffee mill and 10 g were added to 50 ml ethanol in a conical flask with stopper. The mixture was let stand for 3 days in a dark place and then filtered through whatman filter paper. The antioxidant capacity of filtrates was determined according applying modified procedure of Brand-Williams *(Brand-Williams et al., 1995)* in which 80% methanol solution is used for the DPPH reaction. The measurement was performed after incubation at 37˚C for 30 minutes. Three replication were done for each oleoresin and ground product sample.

3.5.2 Measurement by DPPH method in ethyl acetate

To determine the antioxidant capacity of oleoresins modified DPPH method was applied *(Tuberoso et al., 2007)*. The method based on TEAC (Trolox) calibration using ethyl-acetate solution. Because of low boiling point of ethyl-acetate the measurement was carried out at room temperature after 1 hour storage.

***Materials*:** Trolox, DPPH, ethyl acetate

Solutions: 0, 001 M DPPH solution (39,4 mg DPPH in 100 ml ethyl acetate).

0,001 M Trolox solution (25,03 mg Trolox in 100 ml ethyl acetate).

***Reagent*:** 10 x diluted DPPH in ethyl acetate.

Blank: 2 ml reagent+ 50 µl ethyl acetate directly to the cuvette and cover.

Sample: 50 µl sample + 2 ml reagent directly to the cuvette and cover.

Trolox calibration: 1, 2, 4, 6, 8, ml of Trolox were taken to a volumetric flask and the volume was adjusted to 10ml, (Trolox+ ethyl acetate= 10 ml). 50 µl was taken from each and 2 ml reagent was added. This reaction took place for 15 minutes.

Samples were incubated for 60 minutes at room temperature in a dark place into the spectrophotometer. At 517 nm we measured the absorbance toward ethyl acetate.(absorbance of blank – absorbance of sample).

3.6. Antimicrobial properties

3.6.1. Antibacterial effect

In the applied antibacterial tests two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, while three Gram-positive bacterium, *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* were used. These bacteria are responsible for food-borne illness and *E. coli* is a human health indicator. *B. cereus* is a spore-forming bacterium. All the tested microbes were obtained from stock cultures at the Department of Microbiology, Central Food Research Institute, Hungary, the tested oil samples are shown in Results and Discussion.

We tested the antimicrobial effect by using agar diffusion method. Brain heart infusion (BHI) soft agar was prepared and inoculated with the 24 hours old culture (shake) of the bacteria, to reach $1x10^8$ cfu / ml final cell count. Plates were poured with 10-10 ml of the inoculated medium into Petri dishes of 9 cm diameter. After solidification wells of 5 mm diameter were made into the agar. 10-10µl of the dilutions were measured into the wells and plates were incubated for 24 or 48 hours at 30°C (*B.cereus, L.monocytogenes* and *Ps.aeruginosa*) or at 35°C (*St.aureus* and *E.coli*). Wells were numbered clock wisely. We measured the diameter of the inhibition zone in mm.

Dimethyl-sulphoxide (DMSO) was used for diluting volatile oils.

For negative and positive control 10µl DMSO and 10µl antibiotic or inhibitory materials were measured to 1-1 well in each Petri dish (Table 4).

(BHI= brain heart infusion)

(cfu= colony forming unit).The experimental design is shown on (Fig. 32) in the appendix

Table 3:Tested microorganisms

Tested microorganisms	
1. *Bacillus cereus*	Gram +
2. Listeria monocytogenes (4ab)	Gram +
3. Staphylococcus aureus (F1347)	Gram +
4. Escherichia coli (B200)	Gram -
5. Pseudomonas aeruginosa (F1087)	Gram -
6. Penicillum vermiculatum	Fungi
7. Aspergillus niger	Fungi
8. Kluyveromices loddeare (F 1347)	Yeast

Table 4: Inhibitory materials used for different microbes

Microorganisms	Inhibitory materials
Bacillus cereus	*Ampicillin 10 mg/ml*
Listeria monocytogenes	*Ampicillin 10 mg/ml*
Staphylococcus aureus	*Cefalosporin 10 mg/ml*
Escherichia coli	*Polymixin B 5000 UI/ml*
Pseudomonas aeruginosa	*Ampicillin 10 mg/ml*
Penicillium vermiculatum	*Potassium-sorbate 1%*
Aspergillus niger	*Potassium-sorbate 1%*
Kluyveromyces loddeare	*Potassium-sorbate 1%*

3.6.2. Antifungal effect

The tested fungi were *Penicillium vermiculatum* (Budapest Corvinus university) and *Aspergillus niger* (Central Food Research Institute, Budapest).

10 ml malt soft agar were put into a Petri dish, and the test fungi were inoculated in the center of the Petri dish (well 7, Figure 32 in the appendix) . When the colony diameter of the test fungi reached 20 mm, 0,5 cm away from the edge of the plate 5 mm wells were made and 10-10µl extract samples were put into them.

3.6.3. Antiyeast effect

The tested yeast was Kluyveromyces Loddeare F 1347

0.5% glucose was added to malt soft agar (MG). We made 72 hours shaked culture from the yeast and plates were poured with the inoculated soft MG agar.The starting cell number at the plate was 10^7/ml. The wells arrangement was like that for bactericidal effect test.

4. RESULTS AND DISCUSSION

4.1 Results on thyme

4.1.1 Pigment composition of thyme

4.1.1.1 Carotenoids

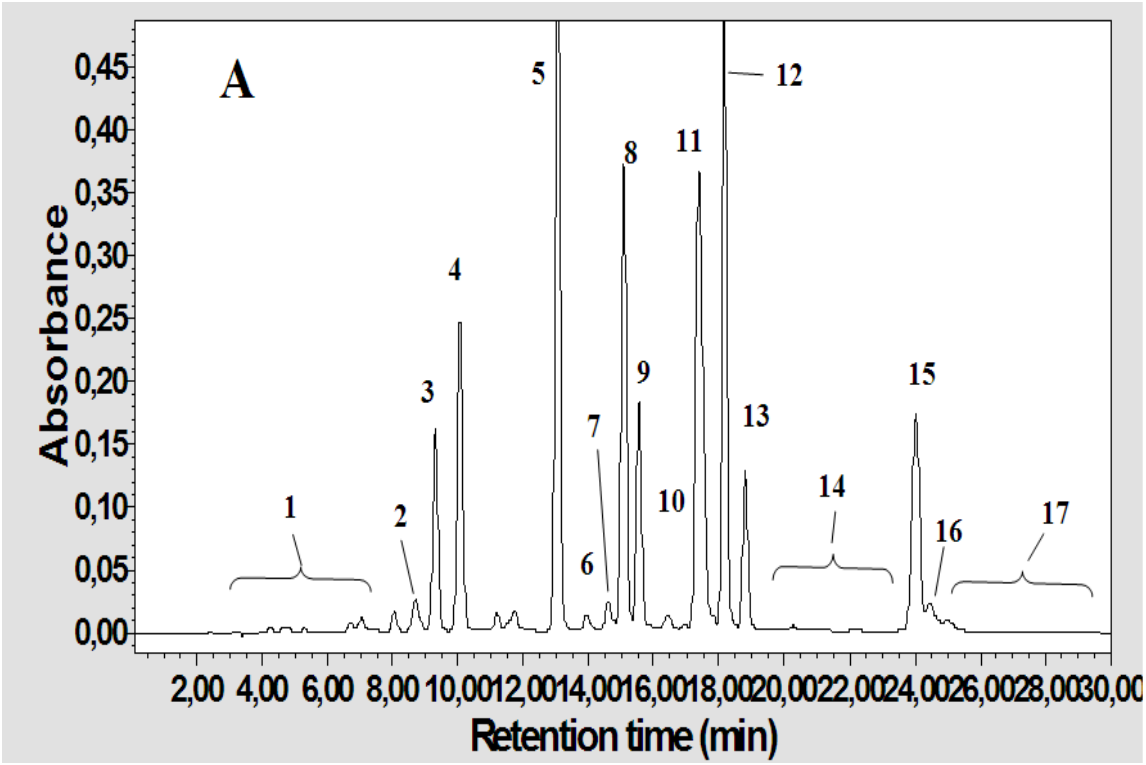
The HPLC analysis of photosynthetic pigments showed that ground thyme leaves contain neuchrom, violaxanthin, lutein, and β-carotene as the major carotenoids with other compounds as the minor ones (Figure 19) .The applied procedure provided good separation of cis isomers of the major carotenoids from their all trans forms. As shown in Table 5 fresh leaves of thyme contain considerably high level of carotenoids particularly those having biological activity such lutein and β-carotene as compared to some other leafy vegetables or herbecial products *(De Sá and Rodrigues, 2003; Serrano et al., 2005*). According to the obtained data 100 g of freshly harvested thyme can supply 2.8 mg lutein and β-carotene that account for 1/6 of the recommended dietary daily intake of carotenoids (15mg) that required for disease prevention.*(Gey et al., 1993).*

Table 5 : Chlorophyll, carotenoid and tocopherol content of fresh, dried at room temperature and 50°C-dried thyme leveas as determined by HPLC methods

Components		Concentration (ug/g dry matter)		
		Fresh raw	Room-dried	50 °C dried
Chlorophylls	Oxidized Ch b	2091±105	679±28	683±48
	Chlorophyll b	768±31	310±12	334±17
	Chlorophyll b'	Tr	86±5	139±11
	Oxidized Ch a	5942± 357	1346±81	1201±72
	Chlorophyll a	1062± 53	632±44	754±45
	Chlorophyll a'	331±23	140±6	131±9
Carotenoids	Neuchrom	323±22	82±5	79±5
	Violaxanthin	606±29	63±4	40±3
	Lutein	1792±72	549±38	553±33
	Neolutein	57± 3	14±1	76±5
	Beta-carotene	1045±63	92±6	103±8
	Cisz-Beta-carotene	118±4	13±1	17±2
Tocopherols	Alfa	351±24	88±7	79±6
	Beta	54± 4	47±4	11±1
	Gamma	248±18	192±13	105±7

Values represent means ± standard deviation N = 3

Because dry and milled thyme is usually used as food ingredient it was important to investigate the effect of drying on its pigment compositionin and content. From the data of Table 5 it is evident that pigment of thyme leaves are susceptible to both natural and artificial drying with violaxanthin and β-carotene being the most sensitive compounds. There was no substantial difference between the two samples, which were dried by different methods, in their carotenoid content except that for violaxanthin, which showed low stability toward artificial drying at 50°C for few hours.



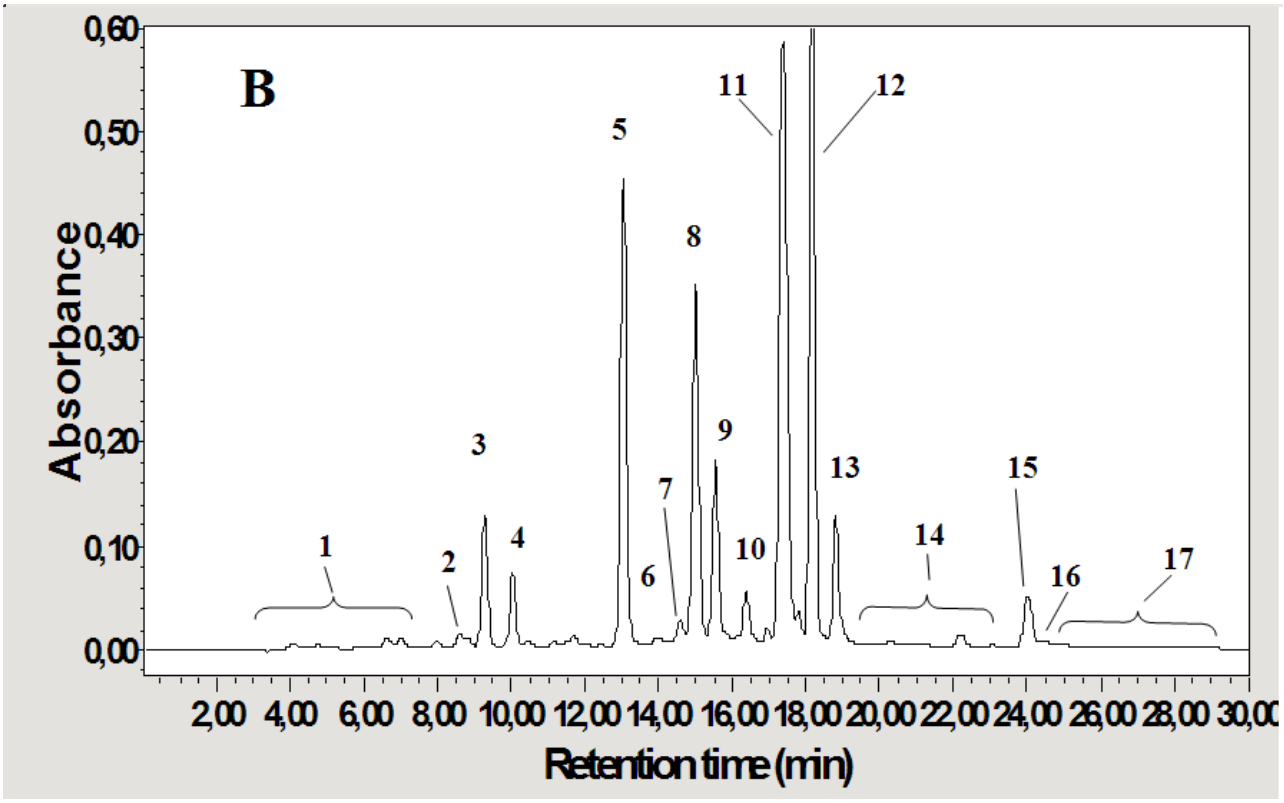


Figure 19. HPLC profile of photosynthetic pigment from fresh(A) and dried (B) thyme seperated on RP-C18 column and eluted with gradient system as described in material and method. .

The numbers seen at the HPLC profile in (Figure 19) means the following:

1: phytyl free 2: neoxanthin; 3: neochrom; 4: violaxanthin; 5: lutein; 6: cis-lutein; 7: oxidized chlorophyll b; 8: chlorophyll b; 9: chlorophyll b′; 10: oxidized chlorophyll a-1; 11:oxiodized chlorophyll a-2; 12: chlorophyll a; 13:chlorophyll a′; 14: pheophytins; 15: β-carotene; 16: cis β-carotene; 17: pyropheophytins

4.1.1.2 Chlorophylls

Among components of green pigments, the leaves contained chlorophyll a and b as well as oxidised derivatives as the major and some phytyl-free chlorophyllides and Mg-free pheophytins as the minor constituents. The presence, of oxidised chlorophylls, even in fresh and dried leaves, is most likely due to high activity of an oxidizing agent existing in such a product.

Like carotenoids, chlorophylls in thyme leaves are very sensitive to both natural and artificial drying with chlorophyll a being the most susceptible one that lost around 78% of its original content as a function of natural drying. It is interesting that the 10 epimer of chlorophyll b (b′) existed at very low concentration as compared to chlorophyll a′, which existed at considerably high level in fresh thyme leaves. Drying process specially at 50°C initiated formation of chlorophyll b′, while chlorophyll a′ tended to significantly decrease (Figure 19).

4.1.2 Tocopherol content

The normal-phase HPLC analysis indicated that thyme oil contain α-, β- and γ- homologues as the main components of tocopherol fraction with α- tocopherol being dominant in the raw materials (Figure 20). The occurrence of vitamin E rises nutritive value and increase stability toward oxidative deterioration of such a product.

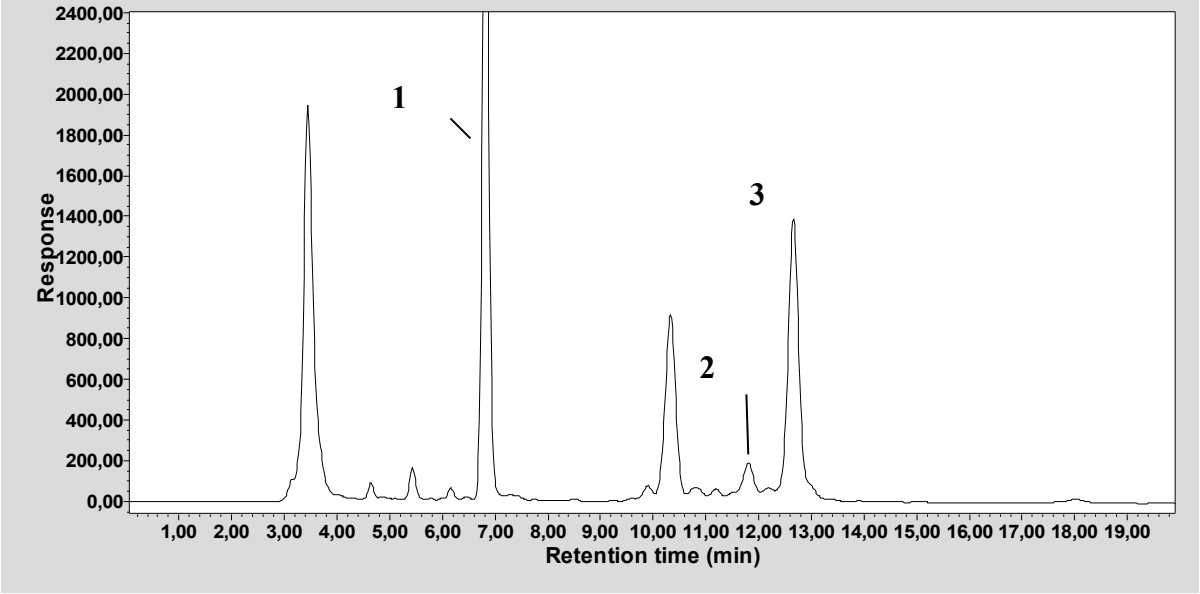


Figure 20. HPLC profile of tocopherols from freshly harvested thyme. Separation was performed on normal-phase column eluted with 99.4:0.6 n-hexane-absolute alcohol. 1: α- tocopherol, 2: β-tocopherol, 3: γ-tocopherol

As a function of natural and artificial drying thyme lost considerable amount of tocopherol mainly α-homologue, the biologically active form of vitamin E, Table 5. The obtained results support the earlier mentioned notice that oxidation processes are activated, to a high extent, during processing of thyme product.

4.1.3 Volatiles of thyme

Figure 21. shows the GC-MS profile of volatiles of thyme leaves extract separated on Capillary column and detected by mass selective detector. The major constituents of the essential oil part of thyme extract were found to be carvacrol, thymol, γ-terpinene and ρ-cymene that are resposible for the intensive odor and therapeutic effect of such herb or medicinal plant. The content expressed as g/100g and % of each compound related to the total area of all peak appeared on the chromatogram is shown in Table 6. The levels found fall in the ranges reported by *(Kulisic etal.,2005)* for the major volatiles of the wild thyme extract. However some variation in the volatile composition could be found between these results and those reported by *Asllani and Toska,2003.* This variation is most likely due to genetic, agricultural and environmental factors.

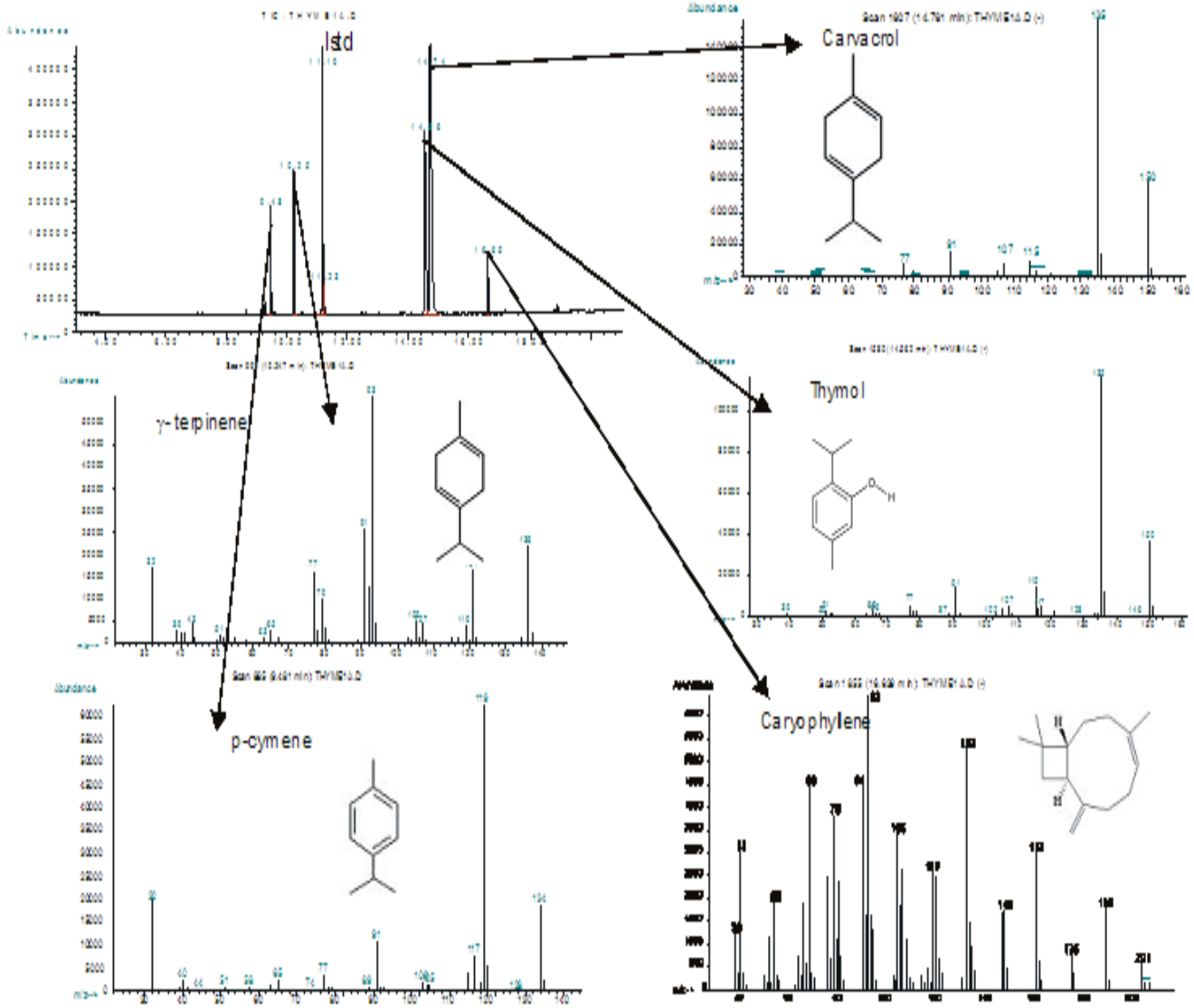


Figure 21. GC-MS profile of volatiles if thyme supercritical extract separated on capillary column and detected by mass selective detector.

Table 6: Composition of volatiles from dried thyme leaves as analysed by GC-MS

Compounds	Retention time	Concentration	
		Percentage % in volatiles	g/100g herb
β-myrcene	8.66	Tr	Tr
A-terpinene	9.3	Tr	Tr
ρ-cymene	9.48	21.2 ± 2.0	0.27 ±0.03
Γ-terpinene	10.25	20.6 ±1.0	0.27 ±0.01
Thymol	14.56	5.5 ±1.2	0.07 ±0.02
Carvacrol	14.74	58.2 ±1.8	0.75 ±0.002
B-caryophyllene	16.65	Tr	Tr
Total	-	100	1.29±0.04

4.1.4 Super-and sub-critical extraction of thyme

4.1.4.1 Supercritical CO_2 extraction

The extraction curves of thyme oleoresin were obtained by plotting solvent use-up (solvent/solid ratio) versus extract yield (g/100g ground thyme). At constant temperature, the applied pressure affected oil solvating capacity of SC-CO_2 (Figure 22). At a constant pressure, the extraction curves can be characterized by an initial steep linear increase of oil solubility as a function of increased volume of solvent passing through the sample in the extractor. Then the curve reaches a plateau and approaches the maximum yield of oleoresin. The tangent of the straight line gives the equilibrium solubility of thyme oil in SC-CO_2 (g extract/100g CO_2).

As a function of pressure rise from 100 to 400 bar, the solubility of oil in SC-CO_2 repeatedly increased. The maximum yield was 5.2 g oleoresin from 100 g ground thyme (but for the second thyme sample used for volatile and microbiological tests and was extracted at the Technical University of Budapest, the maximum yield was 2.44 g). Under the used conditions the ratio of solvent/thyme needed for the complete recovery of the oil was approximately 6, which is moderate as compared to the 8-11 found for SFE of paprika using the same solvent and similar extraction conditions *(Daood et al., 2002).*

4.1.4.2 Sub-critical propane extraction

(Figure 22) also shows the extraction curve of thyme oleoresin by propane at sub-critical conditions. The relationship between yield and solvent use could be characterised by a steep linear increase followed by a steady state to give the maximum yield. From the tangent of the linear relationship, the initial equilibrium solubility was estimated to be 5.1 g extract/100 g propane. This value is well above the 2.8 found with CO_2 at 35°C and 400 bar. The extraction curves at 30 and 50 bar run close to each

other indicating that complete extraction of oleoresin by propane can be approached even at low pressures. The maximum oleoresin yield with propane was 5.8g/100 g thyme.. The difference between SC-CO_2 and sub-critical propane, which has special economic importance, was in the solvent use-up that was about 3 times lower with propane. In addition, color intensity of extracts (visually) obtained by propane extraction was greater than that of the SF-CO_2.

It was found that SC-CO_2 and sub-critical propane can hardly extract chlorophylls from ground thyme leaves. Substantial artefact formation is expected as a function of supercritical or sub-critical fluid extraction of plant products rich in photosynthetic pigments. Although solubility of some chlorophyll derivatives in SC-CO_2 could be improved by increasing the pressure or temperature of the extraction, the achieved increase in the level of these constituents was not so high to produce satisfactory chlorophyll-rich oleoresins or oils. The use of suitable modifiers that assist in dissociation of chlorophylls and carotenoids from the tissues and to increase their partitioning in the solvents under super- and sub-critical fluid extraction conditions is recommended to produce oleoresin with minimum artefacts from such a product.

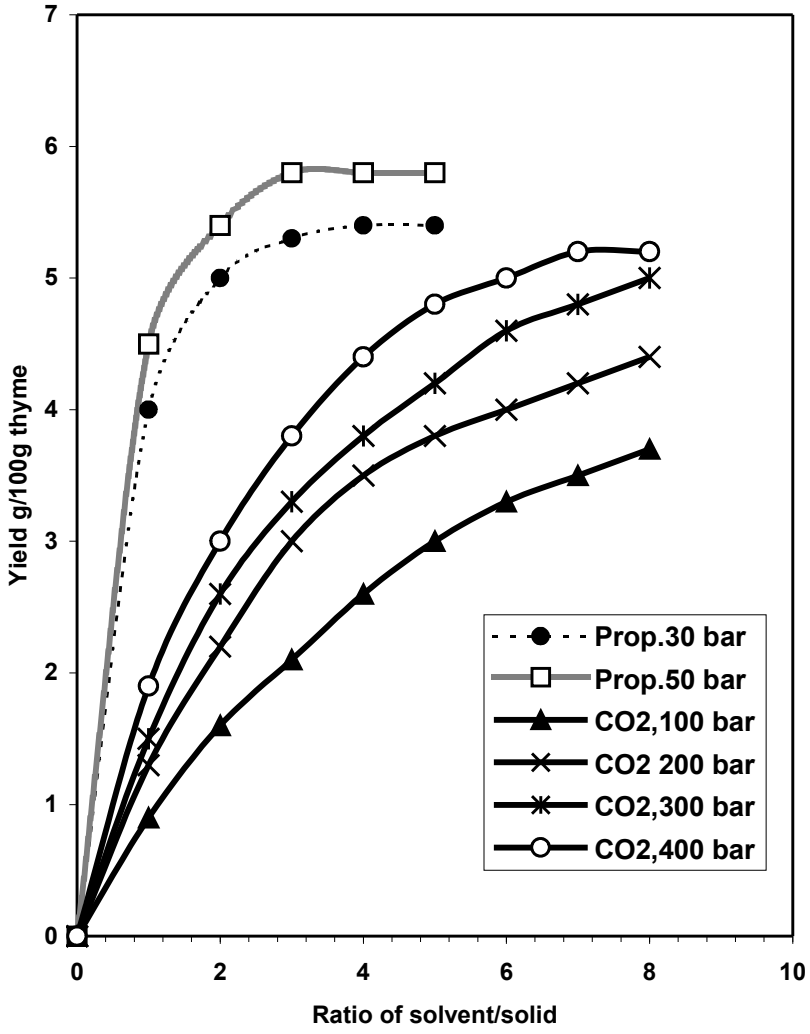


Figure 22. Effect of pressure and temperature on supercritical and sub-critical extraction

4.1.4.3 Effect of extractions on pigments, tocopherol and volatiles

On Carotenoids

Table 7 and 8 show the content and composition of carotenoids in the residues and oleoresin as affected by the extraction methods and conditions used. Performance of SFE at 100 bar CO_2 extracted hardly any of the pigments from ground leaves. Increasing the pressure from 100 to 400 bar at each temperature applied caused the amounts of recovered carotenoids to substantially increase in the oleoresin fraction. However, the recovered amount did not exceed, at the best case, 82% of the initial content of carotenoids in the starting raw materials. The value of 81.8% recovery was recorded for cis

- β-carotene in oleoresin obtained using SF-CO_2 at 400 bar and 55°C. It is to be mentioned that further izomerization of β-carotene may be contributed to the high value recorded for cis-β-carotene in the extract under the conditioned applied in the extraction process. Solubility in SF-CO_2 of all the carotenoids examined was significantly lower at 35°C than at 55°C (p =0.01-0.001) applying the same pressure. These results indicated that association of the different carotenoids, particularly oxygen containing polar xanthophylls, with the membranes of chloroplasts in thyme leaves is stronger than the solvating power of SF-CO_2 under the conditions used in the experiment. In addition polarity of carotenoids affected to a high extent their solubility in such a solvent with the less polar ones being of higher solubility. The maximum recovery of β-carotene, the only vitamin A precursor in thyme leaves was 50.8% as related to the initial content of raw materials before extraction.

As a function of pressure and temperature, all trans to cis isomerisation of carotenoid compounds (mainly lutein in the residues) was noticed. The concentration of cis isomers increased parallel to the increasing pressure irrespective of the temperature applied. The concentration of cis lutein increased from 4 μg/g in the starting material to 64 μg/g in the residues after extraction at 400 bar and 35 °C. Similar change has been previously mentioned for carotenoids from spice red pepper (paprika) extracted under similar conditions (*Daood et al., 2002*). This may be due to initiation of all-trans to cis isomerization of carotenoids under the conditions used in the SFE and/or to the fact that partitioning of Z-isomers in super- and sub-critical fluids is much higher than that of all-E compound (*Eggers et al., 1999*). These results also agree with those of Tuma and Schneider (*Tuma and Schneider, 1999*), who studied the changes in isomerisation of carotenoids as a function of time, pressure and temperature in a static solubility mode using SC-CO_2. The authors stated that pressure dependency of isomerisation was between 200 and 400 bar and at higher pressure (600 bar) the formation of cis isomer decreases.

In case of cis-β-carotene, the highest level was estimated in residues remained after SFE at 100 bar. Unlike that of cis-lutein, the level of cis-β-carotene was close to that of the starting materials. With pressure rise to over 100 bar the content of the isomer tended to decrease. Since the total of the amount remaining in the residues and that recovered in the oleoresin is not equal to (lower than) the amounts found in the starting materials cis isomers undergo some degradation during extraction process. This holds true for other carotenoid compounds with violaxanthin showing the greatest destruction as a function of extraction conditions. The lowest loss of carotenoids was recorded with extraction at 100 bar and 35°C.

It was also found that solubility of cis isomers of both lutein and β-carotene in SC-CO_2 was significantly improved by increasing the extraction temperature to 55°C.

With sub-critical propane, the pigment recovery ranged between 0,7% for lutein and 66% for β-carotene with the less polar fractions being the easier to extract. This can be confirmed by the high amounts of polar xanthophylls and the low amounts of β-carotene remained in the residues after subcritical propane extraction.Sub-critical propane has been found to be superior over SC-CO_2 in the extraction of less polar diesters of carotenoids from spice paprika *(Illés et al.,1999; Daood et al., 2002).*

Table 7: Effect of extraction conditions on the carotenoid content of oleoresin and residues extracted from ground thyme leaves at different conditions

Extraction conditions			Carotenoid concentration µg/g powder				
P. bar	***Temp. °C***	***Solvent***	***Violaxan-thin***	***Lutein***	***Cis-lutein***	***β-carotene***	***Cis-β-carotene***
In residues							
100	**35°C**	**CO_2**	**22.3±1.8**	**154.3±12.3**	**20.1±1.8**	**39±2.7**	**13±1.2**
200	**35°C**	**CO_2**	**19.2±1.2**	**119.2±8.3**	**34.3±2.4**	**17±1.1**	**5±0.6**
300	**35°C**	**CO_2**	**17.6±1.5**	**114.8±7.0**	**44.8±4.1**	**16±1.4**	**3±0.2**
400	**35°C**	**CO_2**	**10.2±0.7**	**98.1±7.8**	**63.7±4.8**	**11±0.9**	**3±0.3**
100	**55°C**	**CO_2**	**11.3±1.2**	**139.8±9.8**	**6.2±0.4**	**31±2.2**	**13±1.1**
200	**55°C**	**CO_2**	**14.7±1.5**	**125.2±13.8**	**34.4±3.1**	**23±2.1**	**6±0.5**
300	**55°C**	**CO_2**	**15.2±1.5**	**112.310.1**	**37.9±4.1**	**12±1.3**	**4±0.4**
400	**55°C**	**CO_2**	**14.1±1.3**	**109.7±8.8**	**55.3±5.1**	**6±0.4**	**3±0.2**
50	**25°C**	**Propane**	**46.8±5.1**	**104.2±7.2**	**66.4±4.6**	**4±0.3**	**Nd**
In oleoresin							
100	**35°C**	**CO_2**	**< 1**	**< 1**	**< 1**	**6.4±0.4**	**Tr**
200	**35°C**	**CO_2**	**2.1±0.1**	**2.3±0.2**	**1.3±01**	**7.2±0.6**	**4.4±0.3**
300	**35°C**	**CO_2**	**4.3±0.3**	**5.4±0.4**	**4.2±03**	**8.4±0.7**	**5.2±0.5**
400	**35°C**	**CO_2**	**7.6±0.8**	**6.1±0.5**	**4.4±0.4**	**12.6±1.3**	**8.6±0.7**
100	**55°C**	**CO_2**	**1.3±0.1**	**1.2±0.1**	**1.1±0.1**	**3.4±0.2**	**1.2±0.1**
200	**55°C**	**CO_2**	**3.0±0.2**	**2.2±0.2**	**1.4±0.1**	**3.7±0.3**	**1.4±0.1**
300	**55°C**	**CO_2**	**8.2±0.7**	**5.8±0.5**	**5.2±0.3**	**13.2±1.3**	**6.2±0.6**
400	**55°C**	**CO_2**	**11.7±1.2**	**8.1±0.6**	**8.6±0.6**	**23.5±1.8**	**12.6±1.4**
50	**25°C**	**Propane**	**1.4±0.1**	**1.3±0.1**	**1.5±0.1**	**30.7±2.7**	**7.5±0.7**
Starting materials (Control) *			**49.2±3.1**	**178.6±9.8**	**4.3±0.3**	**46.2±4.1**	**15.4±1.1**

nd = not detected, tr= traces

***** The control was prepared by extracting the ground thyme by a mixture of 1,2-di-chloroethane acetone- methanol (2:1:1)

Table 8: Percentage % (as related to content in starting material) of remained and in-oil recovered carotenoids of thyme extracted with supercritical CO_2 and sub-critical propane under different conditions.Data derived from Table 7.

Extraction conditions			Carotenoid concentration %				
Pressure Bar	***Temp. °C***	***Solvent***	***Violaxanthin***	***Lutein***	***Cis-lutein***	***β-carotene***	***Cis-β-carotene***
In residues							
100	**35°C**	**CO_2**	**45**	**86**	**465**	**84**	**85**
200	**35°C**	**CO_2**	**39**	**67**	**797**	**37**	**32**
300	**35°C**	**CO_2**	**36**	**64**	**1042**	**35**	**19**
400	**35°C**	**CO_2**	**21**	**55**	**1481**	**24**	**19**
100	**55°C**	**CO_2**	**23**	**78**	**144**	**67**	**84**
200	**55°C**	**CO_2**	**29**	**70**	**796**	**50**	**39**
300	**55°C**	**CO_2**	**30**	**63**	**814**	**26**	**26**
400	**55°C**	**CO_2**	**29**	**61**	**1286**	**13**	**19**
50	**25°C**	**Propane**	**95**	**59**	**1544**	**9**	**Udl**
In oleoresin							
100	**35°C**	**CO_2**	**Udl**	**udl**	**udl**	**14**	**Tr**
200	**35°C**	**CO_2**	**4,3**	**1,3**	**30**	**16**	**28**
300	**35°C**	**CO_2**	**8,7**	**3, 0**	**98**	**18**	**34**
400	**35°C**	**CO_2**	**15,5**	**3,4**	**101**	**27**	**56**
100	**55°C**	**CO_2**	**2,6**	**0,7**	**25**	**7**	**8**
200	**55°C**	**CO_2**	**6,1**	**1,2**	**33**	**8**	**9**
300	**55°C**	**CO_2**	**16,6**	**3,2**	**121**	**29**	**40**
400	**55°C**	**CO_2**	**23,7**	**4,5**	**200**	**51**	**82**
50	**25°C**	**Propane**	**2,8**	**0,7**	**33**	**66**	**49**
Starting materials (Control) *			**100**	**100**	**100**	**100**	**100**

Udl: under detection limit

Tr : traces

* The control was prepared by extracting the ground thyme by a mixture of 1,2-di-chloroethane acetone- methanol (2:1:1).

Like SC-CO_2, sub-critical propane promoted all trans to cis isomerisation of lutein. It is worthy to mention that in both extraction methods and under the different conditions used the sum of each carotenoid studied in oleoresin and residues was less than the content in the starting materials revealing the marked loss of carotenoids during extraction process as a result of destruction to undetectable molecules.

On chlorophylls

Table 9 summarises the changes on the content of different chlorophylls as a function of SC-CO_2 and sub-critical propane extraction. At both temperatures applied, there was a remarkable increase in the content of phytyl-free derivatives (chlorophyllides and pheophorbides) as also seen in (Figure 23). These results agree with earlier report that emphasised the hydrolysis of different bio-polymers during extraction with compressed gases (solvents) *(Wang et al., 2004).* It seems that the hydrolysis of chlorophylls is temperature-independent since it occurred at both temperature applied during the SFE.

Significant decrease in the content of chlorophyll b (Figure 24) and chlorophyll a (Figure 25) with a proportional increase of the content of oxidised derivatives in the residues as a result of extraction by both SC-CO_2 and sub-critical propane As the activation of biochemical factors such as oxidizing enzymes, is not expected due to low water content of the dried thyme leaves it is believed that an interaction between chlorophylls and some electron accepting or oxygen donning endogenous agents such as oxidizing phenols may be behind the subsequent accumulation of oxidized chlorophylls occurred during the SFE process. Thyme has been reported to contain high level of aromatic and non-aromatic phenols that may have antioxidant or pro-oxidant properties depending on the conditions under which they exists and function *(Martin et al., 2003; Wang et al., 2004).*

Epimerisation of chlorophylls (formation of b′ and a′, the 10-epimers of chlorophylls) was also activated, by increasing the pressure. With extraction at 35°C the increase in both chlorophyll a′ and b ′was significantly higher than that estimated at 55°C ($p = 0.01$).

In oil, even with propane extraction, and despite the increase in solubility by increasing the pressure, traces or small quantities of chlorophyll derivatives could be detected and quantitatively determined. The brownish green pigment consisted of mainly pheophytins with pheophytin a being the major compounds. Since pyropheophytin a could not be detected in the starting material and propane-extracted oleoresin it is believed that this derivative is formed as a function of high pressure and temperature applied in the SFE process and immediately dissolved in supercritical CO_2. Some physical factors such as high pressure and temperature in canning and blanching of green vegetables have been reported to initiate epimerisation on chlorophyll molecules *(Daood, 1993).*Recovery of different pheophytins in SC-CO_2 was increased proportionally to the increase of pressure at both temperatures

applied. Like carotenoids, chlorophylls lost some of their content as a result of destruction to undetectable compounds during extraction at sub- and supercritical conditions.

It could be concluded that SC-CO_2 and sub-critical propane can hardly extract chlorophylls from ground thyme leaves. Substantial artefact formation is expected as a function of supercritical or sub-critical fluid extraction of plant products rich in photosynthetic pigments. Although solubility of some chlorophyll derivatives can be increased by increasing the pressure or temperature of the extraction, but not to high values, with which a satisfactory chlorophyll-rich oleoresin or oil can be produced.

Table 9: Effect of extraction conditions on the chlorophyll content (μg/g powder) of residues and oleoresin from ground thyme leaves extracted under different conditions

Pigments	SC-CO_2								Propane	Starting material*
	35°C				55°C					
	100 bar	200 bar	300 bar	400 bar	100 bar	200 bar	300 bar	400 bar		
In residues										
Phytyl-free	6.1±0.48	8.2±0.74	9.5±0.86	15.0±1.65	3.8±0.28	10.2±0.62	13.1±1.05	9.2±0.65	11.2±0.44	4.2±0.26
Oxidised Ch.b	2.6±0.12	12.0±0.61	12.6±0.88	14.0±1.26	1.4±0.08	6.2±0.43	11.7±0.86	17.5±1.28	7.1±0.50	1.1±0.10
Ch.b	37.2±2.23	23.8±3.24	13.6±0.81	4.4±0.28	39.1±2.87	27.2±2.17	21.5±1.80	18.1±1.76	11.2±0.56	41.3±3.72
Ch.b´	3.2±0.27	5.1±0.41	5.4±0.41	6.8±0.73	3.4±0.31	3.8±0.40	4.5±0.44	4.8±0.58	3.3±0.21	2.4±0.14
Oxidised Ch.a	6.6±0.32	21.1±1.81	29.2±2.34	47.3±0.61	3.5±0.22	19.6±1.78	16.1±1.57	15.2±1.30	38.3±2.70	6.3±0.38
Ch.a	64.0±4.47	51.5±4.42	41.6±4.68	32.4±2.27	71.2±0.78	59.4±5.44	55.2±4.14	51.0±5.61	41.5±2.86	75.1±7.26
Ch.a´	2.1±0.10	3.3±0.12	4.6±0.32	5.4±0.32	2.4±0.12	3.0±0.24	3.3±0.21	3.9±0.31	3.5±1.14	2.8±0.17
Ph.b	13.4±0.81	12.9±1.16	11.9±0.72	10.5±0.92	13.3±1.22	12.4±0.78	12.8±1.03	12.3±1.12	10.3±0.72	15.0±0.91
Ph.a	55.3±3.87	56.0±3.36	50.2±4.52	51.1±3.58	61.0±5.31	58.8±3.64	61.7±3.77	59.5±5.71	43.3±2.60	65.3±4.58
P.Ph.b	11.9±0.35	8.5±0.81	8.6±0.47	7.7±0.62	14.0±1.04	10.2±0.91	10.1±0.77	8.7±0.66	6.7±0.52	12.4±0.51
P.Ph.a	Tr	Tr	Tr	Tr	2.2±0.13	0.5±0.04	0.8±0.06	0.4±0.02	Nd	Nd
In oleoresin										
Phytyl-free	0.11±0.01	0.21±0.01	0.32±0.02	0.31±0.02	Tr	Tr	tr	Tr	Tr	
Ch.b	Tr	0.32±0.22	0.62±0.04	0.77±0.03	Tr	0.63±0.04	1.65±0.08	2.43±0.14	Tr	
Ch.a	0.13±0.01	0.31±0.21	0.43±0.03	0.63±0.03	0.11±0.01	1.22±0.06	1.81±0.13	2.41±0.15	0.35±0.01	
Ph.b	0.24±0.02	0.64±0.48	0.81±0.06	1.12±0.08	0.13±0.01	0.44±0.03	1.03±0.09	1.23±0.08	1.82±0.12	
Ph.a	1.15±0.08	2.62±0.16	3.33±0.28	3.84±0.27	0.94±0.05	1.42±0.06	1.72±0.13	2.41±0.12	8.83±0.44	
P.Ph.b	0.12±0.01	0.33±0.01	0.56±0.04	0.63±0.02	0.27±0.01	0.71±0.05	1.84±0.14	2.55±0.18	1.67±0.13	
P.Ph.a	0.26±0.02	1.54±0.11	2.13±0.11	2.40±0.21	0.35±0.02	2.36±0.14	5.90±0.25	18.81±1.13	0.53±0.04	

*The control was prepared by extracting the ground thyme by a mixture of 1,2-di-chloroethane- acetone metmethanol (2:1:1). Ch. = Chlorophyll, Ph. = Pheophytin, P.Ph. = Pyropheophytind = not detected tr= trac

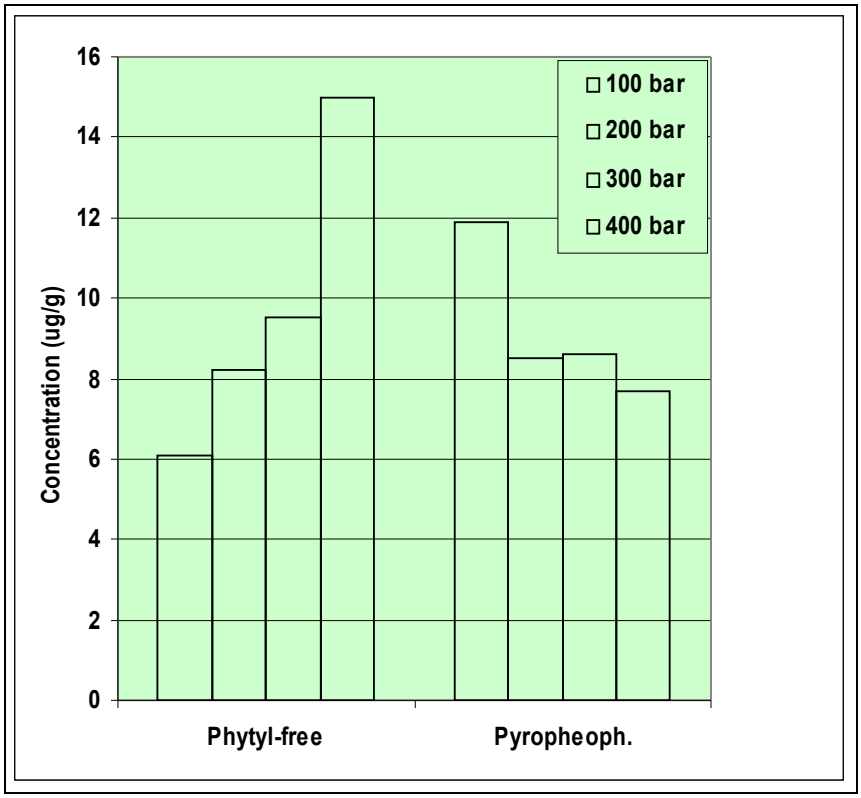


Figure 23. Changes in the chlorophyll derivatives content of residues after SFE of thyme as a function of extraction pressure at 35°C.

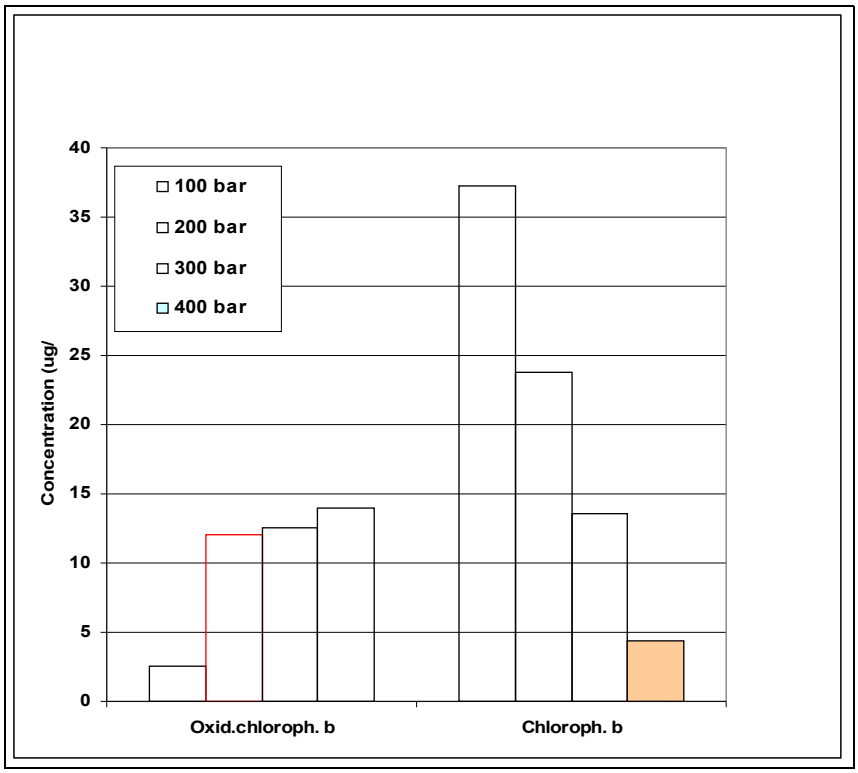


Figure 24. Changes in the chlorophyll b content of residues after SFE of thyme as a function of extraction at 35°C.

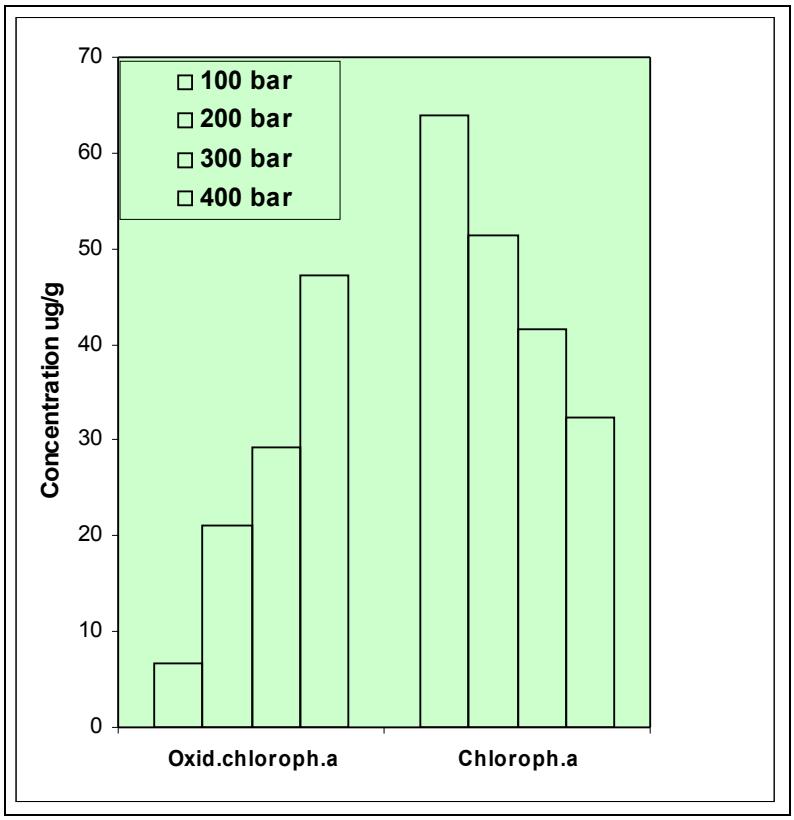


Figure 25. Changes in the chlorophyll a content of residues after SFE of thyme as a function of extraction pressure at 35°C.

The use of suitable modifiers that assist in dissociation of chlorophylls and carotenoids from the tissues and to increase their partitioning in the solvents under super- and sub-critical fluid extraction conditions is recommended to produce oleoresin with minimum artefacts from such a product.

On tocopherol content

Fat-soluble tocopherols are among the effective antioxidants in thyme extracts providing prevention against oxidative degradation and rising up the nutritive and therapeutic value of the extracts as well.

As fat-soluble constituents occurring in the lipid phase of the plant matrices, it was expected that they show behavior similar to that of carotenoids during extraction processes. Table 10 shows the results obtained from supercritical CO_2 and sub-critical propane extractions. The data indicated that 200 bar is the lowest level of pressure needed to recover substantial amounts of tocopherols from ground thyme leaves. At 100 bar the extractability of tocopherols in SC-CO_2 is low particularly at high temperature

(55°C) when low pressure and high temperature are the reason for the low density of the liquid CO_2. The low density of the solvent at 55°C and 100 bar caused the yield of oleoresin to be extremely low. To avoid such problem extraction at around 100 bar should be performed at low temperature to keep the solvent density at convenient level.

When the pressure was raised over 100 bar the content of all of the tocopherol homologues increased in the extracts with slight differences (not significant at p= 0.05) between samples obtained with extraction at 300 and 400 bar. In general extraction at 55°C and pressure higher than 200 bar improved the tocopherol content of the extracts as compared to extraction at 35°C except that for β-tocopherol, which showed a slight, but not significant (p=0.05) decrease. Similar results have been reported for super- and sub-critical extraction of spice paprika *(Illés et al.,1999 ; Daood et al.,2002).*

When calculated on the basis of micrograms recovered from 1 g raw material (ground thyme), as shown in Table 10, there was a great increase in the solubility of tocopherol in SC-CO_2 as a function of pressure increase over 100 bar particularly at 55°C, which is needed to approach the highest recovery of vitamin E (α-tocopherol**).** According to such data the recovery of α-tocopherol ranged between 19% and 75% at 35°C and 2.8% and 90% at 55°C. This supported that to increase recovery of vitamin E in the oleoresins it is necessary to perform SFE at high pressure and temperature. This does not hold true with β- tocopherol and γ- tocopherol. The maximum recovery of the later compounds was recorded with 200-400 bar at 35°C and 55°C. However, β- tocopherol showed underwent some degradation at the highest pressure applied in this work.

Table 10: Changes in tocopherol content of thyme oleoresin as a function of supercritical CO_2 and sub-critical propane extraction conditions

Extraction conditions	*Tocopherol homologues*								
	α-tocopherol			β- tocopherol			γ- tocopherol		
	µg/g oleoresin Mean ± SD	µg/g raw	Reco -very %	µg/g oleoresin Mean ± SD	µg/g raw	Reco -very %	µg/g oleoresin Mean ± SD	µg/g raw	Reco -very %
35°C									
100 bar, CO_2	**505 ± 26**	**16,7**	**19**	**84 ± 4**	**2,8**	**9**	**269 ± 11**	**8,9**	**7**
200 bar, CO_2	**1026 ± 72**	**51,5**	**59**	**174 ± 12**	**8,5**	**27**	**331 ± 19**	**16,2**	**13**
300 bar, CO_2	**1267 ± 76**	**60.2**	**68**	**130 ± 9**	**6,5**	**20**	**265 ± 16**	**13,3**	**11**
400 bar, CO_2	**1290 ± 78**	**65,7**	**75**	**139 ± 11**	**7,1**	**22**	**254 ± 15**	**12,9**	**10**
55°C									
100 bar, CO_2	**358 ± 24**	**2,5**	**3**	**99 ± 6**	**1,6**	**5**	**218 ± 17**	**1,6**	**1**
200 bar, CO_2	**1035± 52**	**51,7**	**59**	**203 ± 12**	**10,1**	**32**	**353 ± 25**	**17,7**	**15**
300 bar, CO_2	**1175 ± 82**	**62,5**	**71**	**205 ± 11**	**10,9**	**34**	**341 ± 24**	**1,3**	**15**
400 bar, CO_2	**1318 ± 92**	**73,4**	**83**	**125 ± 9**	**6,9**	**21**	**355 ± 22**	**19,8**	**16**
25°C									
30 bar propane	**1328 ± 81**	**74,7**	**85**	**243 ±12**	**14,1**	**44**	**377 ± 27**	**21,3**	**17**
50 bar propane	**1371 ± 83**	**79,3**	**90**	**251 ±14**	**15,0**	**47**	**397 ± 29**	**23,7**	**19**
Starting material	**--**	**88 ±7**	**100**	**--**	**32 ±3**	**100**	**--**	**124 ± 9**	**100**

On volatiles

The changes in the major volatiles constituent of thyme oleoresin as a function of pressure in the SFE using CO_2 at 35°C-40°C are shown in Table 11. Because the content of most of volatiles detected in the GC-MS analysis can be influenced by changing the pressure of SFE, the percentage of the major compounds does not show the real quantitative changes taking place as a function of changes in the extraction conditions. Nevertheless, some tendencies of changes can be seen e.g. increase of β-myrcene, ρ-cymene and thymol-proportion and decrease in the content of the other volatiles. It is evident that pressure arround 300 bar is the most convienient for the extraction of carvacrol by SC-CO_2 under the conditions used. The results of quantitative determination of the volatiles from thyme oleoresins using IS (internal standard) are shown in Table 12. The volatiles detected as traces in raw material as shown in Table 6 could be quantitatively detected in the oleoresins as minor constituents.

Solubility of carvacrol and some other volatiles were improved by increasing the extraction pressure from 100 to 300 bar which was the best for achieving the highest level of such phenolic volatiles in thyme oleoresin. Rising pressure to 400 bar or even over did not result in a significant change in the solubility of those volatiles. Thymol and carvacrol were inversely changed while content of thymol decreased, the content of carvacrol increased as a function of pressure raise from 100 to 300 bar. Since no great variation is expected between the detected volatile solubility in SC-CO_2, it is believed that pressure-initiated isomerization reactions may be responsible for an interchange of carvacrol and thymol to other phenolic volatile compounds (positional isomers).

Table 11: Effect of supercritical extraction at different pressure level on the composition and percentage of volatiles from thyme extra

Compounds	**ret. Time**	**Pressure of SFE**		
		100 bar	**300 bar**	**450 bar**
	Min	**Mean**	**Mean**	**Mean**
β-myrcene	**8,66**	**0,41 ± 0,02**	**0,58 ± 0,02**	**0,61 ± 0,00**
α-terpinene	**9,3**	**0,87 ± 0,04**	**0,75 ± 0,17**	**1,02 ± 0,26**
p-cymene	**9,48**	**7,19 ± 0,36**	**8,22 ± 1,02**	**9,89 ± 0,58**
γ-terpinene	**10,25**	**9,37 ± 0,64**	**8,14 ± 0,55**	**10,19 ± 0,66**
Thymol	**14,56**	**18,60 ± 2,45**	**7,57 ± 0,90**	**7,49 ± 0,63**
Carvacrol	**14,74**	**59,45 ± 5,08**	**71,15 ± 5,12**	**67,41 ± 0,63**
B-caryophyllene	**16,65**	**3,90 ± 0,07**	**2,54 ± 0,06**	**3,04± 0,36**
Total		**100,00±9,69**	**100,00 ± 6,21**	**100,00± 0,55**

Table 12: Effect of supercritical extraction at different pressure level on the composition and content (g/100g oleoresin) of volatiles from thyme extracts

Compounds	ret. Time	100 bar	300 bar	450 bar
		Average	Average	Average
	Min	g/ 100g	g/ 100g	g/ 100g
β-myrcene	8.66	0.29 ± 0.01	0.44 ± 0.01	0.36 ± 0.01
α-terpinene	9.3	0.61 ± 0.03	0.57 ± 0.13	0.59 ± 0.15
p-cymene	9.48	5.10 ± 0.25	6.29 ± 0.78	5.74 ± 0.33
γ-terpinene	10.25	6.65 ± 0.45	6.23 ± 0.42	5.91 ± 0.38
Thymol	14.56	13.20 ± 1.74	5.79 ± 0.69	4.35 ± 0.37
Carvacrol	14.74	42.17 ± 3.60	54.43 ± 3.92	39.11 ± 0.36
B-caryophyllene	16.65	2.77 ± 0.05	1.94 ± 0.04	1.76 ± 0.21
Total		70.93± 6.87	76,.49 ± 4.75	58.01 ± 0.32

4.1.5. Antioxidant capacity of thyme products

As shown in Table13 antioxidant capacity of the raw materials and oleoresins was determined using DPPH in methanol method as described in Materials and Methods. The scavenging activity of the dried leaves of thyme was high enough to make this crop receive an interest as a product of high antioxidant activity and of marked functional properties as well. As compared to values recorded for cardamom seeds the average value of 11,6-12,1 found for thyme leaves is significantly higher.

Table13: Antioxidant capacity of the raw materials and oleoresins of thyme

Samples	Extraction conditions	TEAC Average	Error %
		mmol/kg	
Dried thyme	Ethyl alcohol	12.1 ± 1.2	9.6
Dried thyme	Diethyl ether	11.6 ± 1.3	11
Thyme oil	SC-CO_2 100bar 40°C	295± 19.3	6.5
Thyme oil	SC-CO_2 300bar 40°C	381 ± 30.1	7.9
Thyme oil	SC-CO_2 450bar 40°C	388 ± 9,3	2,4

The antioxidant capacity of oleoresin produced by SFE was remarkably high. It is more than 20 times higher than that estimated for oleoresins from cardamom produced under similar conditions. The high antioxidant capacity of such extracts may be attributed to the high solubility of phenol antioxidants occurring in thyme leaves in SC-CO_2 and to the high radical scavenging activity of the essential oils that was concentrated in the oleoresin *(Kulisic et al., 2005).*

4.1.6. Antibacterial properties of thyme oleoresin extracted by SC-CO_2

The antimicrobial properties of herbs rich in volatile oil and especially herbs in *Lamiaceae* family are well known since thousand years. Thyme belong to Lamiaceae plant family and its essential oils possess strong antibacterial, antifungal and antiviral activities. The base of my experiments and research was set on the fact of that no available literature exists about these properties of the more chemically complex extracts obtained by SFE or alcoholic extraction. Therefore, antimicrobial tests were tried and applied to determine the antimicrobial activities of these above-mentioned extracts. In my results the antimicrobial properties of essential oil, ethanolic and SFE extracts of thyme and caradoamom are explained.

Table 14 shows the properties of thyme oleoresins extracted by SC-CO_2 at three different pressures 100 bar, 300 bar, and 450 bar at a temperature of 40°C on the tested bacteria. The thyme oleoresin was diluted 5 times (2, 4, 8, 16, 32 times) as it was very concentrated and difficultly diffuses in the water-based agar.

All the oleoresins of thyme extracted showed inhibition on *B.cereus,* but no significant inhibition change is seen between them. It is also seen that 4x and 8x dilutions showed the best inhibitions against all the examined microbes. This means that the active substances in oleoresins of thyme need dilution to diffuse easily in the agar.

Also, it can be seen that the inhibition on *L.monocytogenes ,St.aureus and E.coli* is similar to that on *B.cereus*, all the three thyme oleoresins showed inhibition except the 32 times diluted sample extracted at 450 bar for *L.monocytogenes.* Diluting the thyme oleoresins helps the active substance for inhibition to diffuse more easily, but also the 4 and 8 times dilutions were the most suitable.

It can also be noticed that no significant changes in inhibition is seen between the different extraction methods. 100 bar is enough to extract thyme oleoresin able for inhibition against *B.cereus, L.monocytogenes* and *St.aureus.*

The 2 times dilution of the thyme oleoresins extracted at 100 bar and 300 bar showed the best inhibition on *Ps.aeruginosa* and by rising up the dilution the inhibition tendency decrease. For oil

extracted at 450 bar the 4 times dilution shows inhibition while the other dilutions did not. This means that the active substance is in very low concentration.

It can be concluded that the active substance responsible for inhibition is found in high concentration and need to be diluted to show its inhibition on the tested microorganisms. Different pressures did not influence significantly its inhibition tendencies. SC-CO_2 is suitable to extract antibacterial compounds from thyme, and 100 bar is enough for this reason. Thyme shows a high antibacterial effect as a herb, so it can be used as food and medicinal industries.

Table14: Antibacterial properties of thyme oleoresins prepared by SFE at 40°C and different pressures

Sample(thyme)	*B.cereus*	*L.monocytogenes*	*St.aureus*	*Ps.aeruginosa*	*E.coli*
100 bar 40°C					
2x dilution	**13.0**	**11.5**	**14.9**	**8.1**	**13.0**
4x dilution	**15.5**	**13.0**	**16.7**	**0**	**17.0**
8x dilution	**18.6**	**13.1**	**17.8**	**0**	**15.5**
16x dilution	**14.8**	**10.5**	**14.6**	**0**	**14.8**
32x dilution	**9.9**	**7.7**	**10.8**	**0**	**11.5**
DMSO	**0**	**0**	**0**	**0**	**0**
300 bar 40°C					
2x dilution	**17.3**	**10.0**	**13.2**	**8.0**	**13.6**
4x dilution	**18.2**	**10.5**	**12.7**	**5.7**	**14.0**
8x dilution	**20.0**	**10.5**	**11.3**	**5.5**	**17.0**
16x dilution	**20.0**	**10.5**	**11.5**	**5.9**	**17.0**
32x dilution	**15.9**	**9.0**	**10.0**	**10.0**	**10.0**
DMSO	**0**	**0**	**0**	**0**	**0**
450 bar 40°C					
2x dilution	**17.0**	**11.5**	**9.2**	**0**	**13.6**
4x dilution	**18.0**	**12.9**	**12.4**	**6.2**	**15.0**
8x dilution	**18.0**	**11.2**	**11.7**	**0**	**14.0**
16x dilution	**17.8**	**11.1**	**11.0**	**0**	**14.7**
32x dilution	**14.9**	**0**	**9.0**	**0**	**9.2**
DMSO	**0**	**0**	**0**	**0**	**0**

4.1.7. Antifungal properties of thyme oleoresins extracted by SC-CO_2

The antifungal effect was investigated as in the case of previous extracts. The thyme extracts were very thick, with very strong odor. The volatiles strongly inhibited the growth of the moulds in the Petri dishes, therefore the effect of dilution could not be evaluated. In the case of *Aspergillus niger* the inhibition was complete. In case of *P.vermiculatum* a very weak growth occurred toward the wells containing 32 x dilution and DMSO. It can be said that *Aspergillus niger* was more sensitive to the

volatile compounds of thyme extract, than *P.vermiculatum*, however more investigations would be necessary for an exact conclusion.

4.1.8. Anti-yeast properties of thyme oleoresins extracted by SC-CO_2

Table 15 shows inhibition of different thyme oleoresins extracted by SC-CO2 at 40°C and at different pressures on *Kluyveromyces Ioddeare* yeast. It can be seen that all three oleoresins showed inhibition but because the oil is very concentrated it was diluted several times. The 2 and 4 times dilutions showed the highest inhibition. Mostly by increasing the dilution ratio, the inhibition tendency decreased. It can be concluded that thyme oleoresin has a good inhibition against *Kluyveromyces Ioddeare* yeast. SF-CO_2 is suitable to extract thyme oleoresin having a good antiyeast affectivity. Changing the SC-CO_2 pressures did not show a significant change in their inhibition effect.

Table 15: Antiyeast properties of thyme oleoresins prepared by SFE at 40°C and different pressures

Sample and SC-CO_2 conditions	Dilution	*Kluyveromyces loddeare*
Thyme oleoresin at 100 bar and 40°C	**2 times dilution**	**13.0**
	4 times dilution	**15.0**
	8 times dilution	**11.8**
	16 times dilution	**11.0**
	32 times dilution	**9.0**
	DMSO	**0**
Thyme oleoresin at 300 bar and 40°C	**2 times dilution**	**16.0**
	4 times dilution	**13.1**
	8 times dilution	**12.5**
	16 times dilution	**9.1**
	32 times dilution	**8.2**
	DMSO	**0**
Thyme oleoresin at 450 bar and 40°C	**2 times dilution**	**14.0**
	4 times dilution	**18.4**
	8times dilution	**14.0**
	16times dilution	**14.0**
	32 times dilution	**9.7**
	DMSO	**0**

4.2 Results on cardamom

4.2.1 Pigment composition of cardamom

Analysis of pigments by HPLC provided separation of the individual carotenoid and chlorophyll constituents of the extracts. Among carotenoids lutein as minor and β-carotene as major compounds could be detected. Our focus was only on β-carotene due to its important role as bio-antioxidant and vitamin A precursor *(Burton and Ingold, 1994).* The HPLC profile consisted of different chlorophyll and Mg-free derivatives (pheophytins). The sum of chlorophyll a and b and that of pheopytin a and b was in the evaluation of the quality attributes of the extracts see (Fig.26).

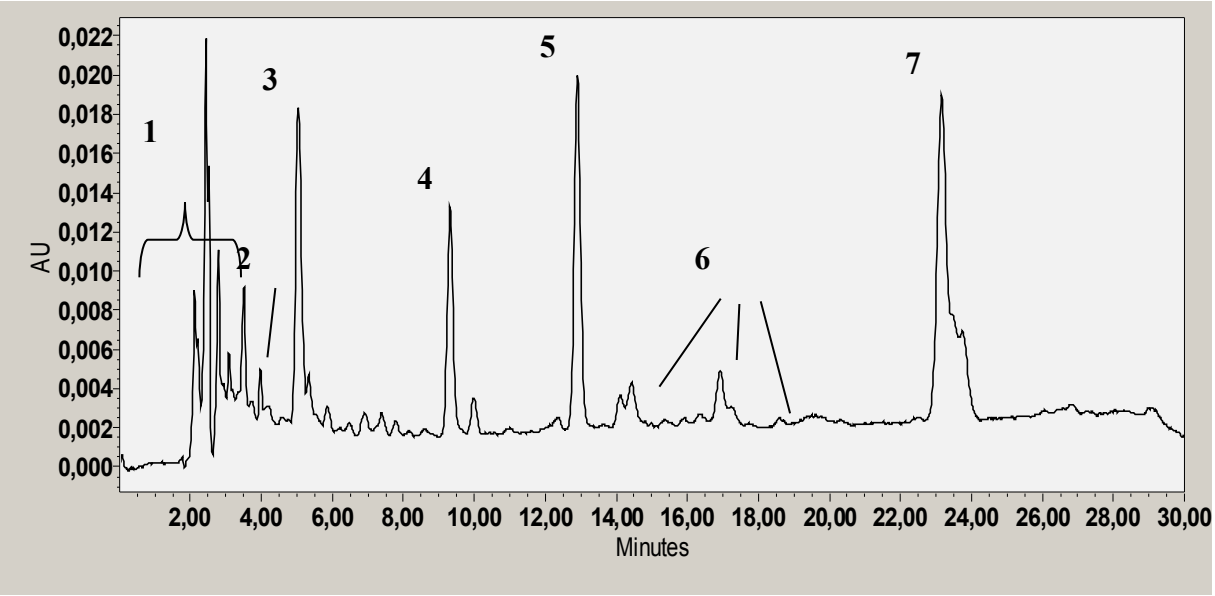


Figure 26. HPLC profile of pigments from cardamom seeds seperated on RP-C18, 5µm, 240x 4,0mm column and eluted with gradient system starting with methanol ending with 10:35:55 methanol-acetonetrile ,isopropanol.Detection at 430nm.1-phytylfree chlorophylls,2-violaxanthin, 3-lutein, 4-chlorophyll b, 5-chlorophyll a, 6- pheophytines, 7-β-carotene.

4.2.2. Tocopherol composition of cardamom

As concerns the antioxidant content of the extracts, the HPLC analysis indicated that cardamom oil contains low level of fat-soluble tocopherols, the components of vitamin E. The major component of the vitamin found in the seeds and extracts was α-tocopherol (the biologically active analogue), while β- and γ- analogues occur as minor constituents see (Fig.27).

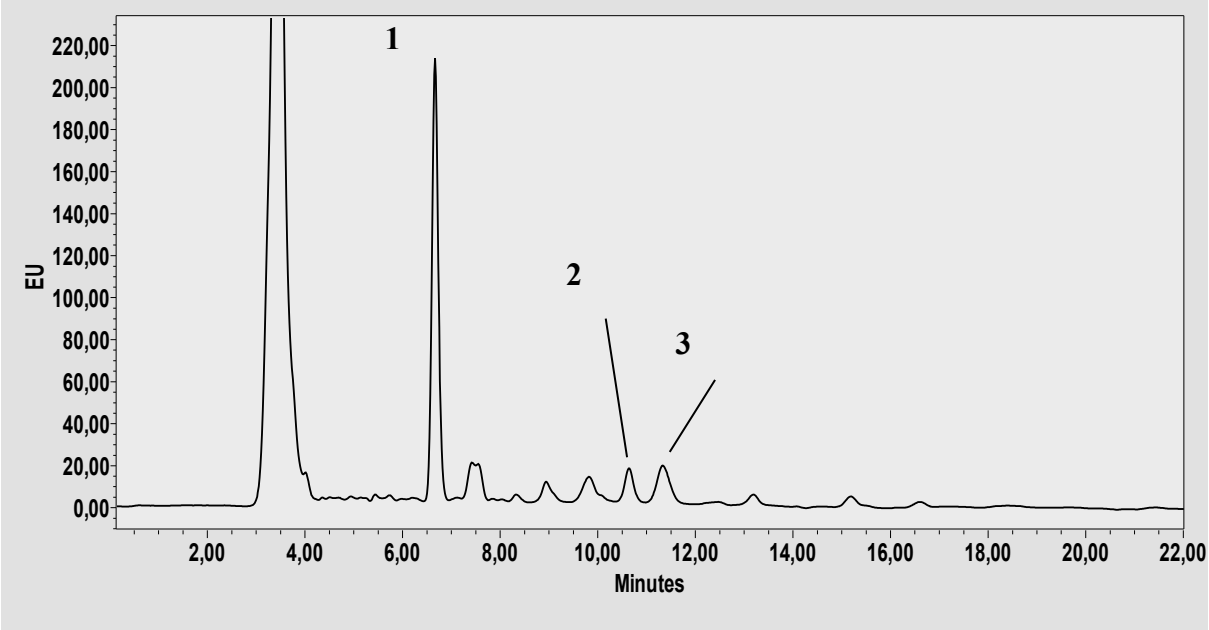


Figure 27. HPLC profile of tocopherols from cardamom seeds seperated on normal- phase silica, 5µm, 240x 4.0mm column and eluted with n-hexane-absolute alcohol(99.4:0.6. Detection at EX: 295nm and EM: 320nm.1-α-tocopherol, 2-β-tocopherol, 3-γ-tocopherol.

4.2.3. Composition of cardamom volatiles

The qualitative analysis carried out by GC-MS showed that cardamom extract contains about 22 volatile compounds with, 1,8-cineol, linalool, neral, linalyl acetate and α-terpinyl acetate being the major ones as shown in Table 19 and Table 20 and (Figure28). These results agrees with those found by Gopalakrishnan *(Gopalakrishnan, 1994)* The low concentration of tocopherols in the seeds and oils of cardamom may stand behind the low storage stability of volatile compounds of such products as stated by the authors.

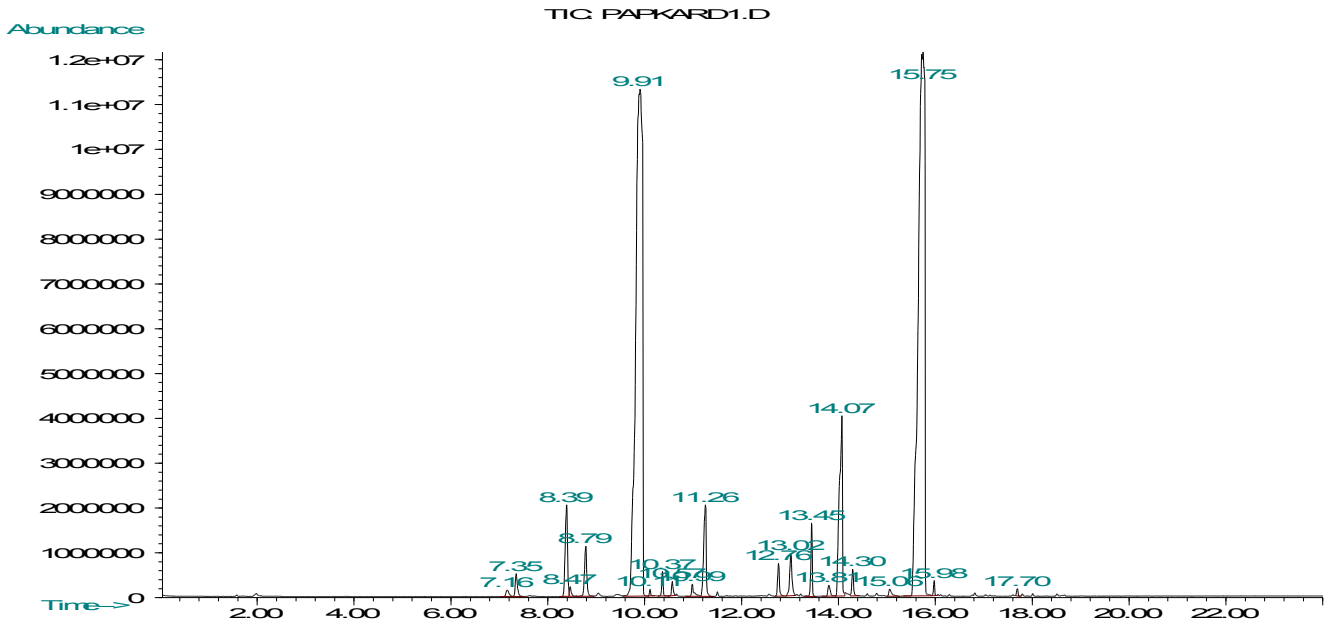


Figure 28. GC-MS chromatogram of volatiles extracted from cardamom seeds. Separation was performed on RH-5ms 30 m x 0.25 mm i.d, 0.25 µm film thickness column. Identification according to retention time shown in Table 19.

4.2.4. Super and sub-critical extraction of cardamom

The extraction curves of cardamom oleoresin were obtained by plotting the solvent/solid ratio versus extract yield (g/100g ground seeds). At a constant temperature, the applied pressure affected oil solvating capacity of SC-CO_2 (Figure 29). At a constant pressure, the extraction curves can be characterized by an initial steep linear increase of oil solubility as a function of increased volume of solvent passing through the sample in the extractor. Then the curve reaches a plateau and approaches the maximum yield of. The tangent of the straight line gives the equilibrium solubility of cardamom oil in SF-CO_2 in terms of g extract/100g CO_2. The values estimated for equilibrium solubility were 7.4, 5.7 and 4.6 when the extraction was performed at 25°C and 100 bar respectively.

As a function of pressure increase from 100 to 300 bar, the solubility of oil in SC-CO_2 repeatedly increased. Applying SFE at 300 bar resulted in no significant increase in the yield of the extract even with contact time of 3h (solvent/solid ratio of approximately 6). The maximum yield approached with SF-CO_2 was 6.65 g extract from 100 g ground cardamom. This value is significantly lower than 7.9 reported by Gopalakrishnan and Narayanan *(Gopalakrishnan and Narayanan, 1991* using SF-CO_2 at 100-600 bar and temperature between 40°C and 60°C, but higher than the 5.5 g/100g reported by

Marongiu and co-workers *(Marongio et al., 2004)* who performed the SF-CO_2 extraction at 90-110 bar and 40°C-50°C. The variation in the maximum yield achieved in different studies is most likely due to variations in the oil content of the raw materials (cardamom seeds) used, not to the applied SFE conditions.

With CO_2 at sub-critical conditions (25°C and 80-100 bar) the maximum yield could be achieved with longer contact time of the solvent with the seeds (3-4h). The value of solvent/solid recorded for the maximum yield was around 2.7 while a value of 2.0 was estimated at the best supercritical extraction conditions (Figure 30). It is of practical importance that the increase of pressure from 80 to 100 bar at sub-critical temperature 25°C caused the yield to significantly increase, but no significant increase was found in the amount of extract with increasing the temperature to 35°C. It is believed that high pressures and temperatures (supercritical conditions) are necessary for the solubility of non-volatile fraction of cardamom oil in SC-CO_2, but marked loss of in the volatile fraction is expected, while sub-critical temperatures have preferable effect on reducing the heat-induced loss of essential oils of cardamom extract.

Under sub-and supercritical conditions the ratio of solvent/solid needed for the complete recovery is significantly lower as compared to the 8-11 found for SFE of paprika using the same solvent and similar extraction conditions *(Daood et al., 2002)*. In a study by Catchpole and co-workers *(Catchpole et al., 2003)* high pressure and ratios of solvent to solids have been found necessary for the extraction of pepper by fluid CO_2 at supercritical conditions.

Figure 31 shows the extraction curve of cardamom oleoresin by propane at sub-critical conditions. The relationship between yield and solvent use could be characterised by a steep exponential increase followed by a steady state to give the maximum yield. Solubility of cardamom oil in sub-critical propane increased with the pressure increase from 20 to 50 bar. From the tangent of the linear relationship, the equilibrium solubility was estimated to be 20.3 and 11.2 g oil/ 100 g propane at pressure of 50 and 20 bar respectively. The maximum yield of oil approached under the given conditions was 7.24 g extract/100 g seeds. This value is significantly higher ($p=0.05$) than the 6.65 found with CO_2 at 35°C and 300 bar. The yield achieved with sub-critical propane even at very low pressure is higher than that obtained with SC-CO_2 at the best conditions. This emphasizes that sub-critical propane is suitable solvent for rapid and efficient extraction of oleoresin from cardamom seeds. Furthermore, taking into consideration the low

ratio of propane/seeds necessary for the complete recovery of the extract (around 1), and the process can be accomplished with relatively short time.

To improve extractability of cardamom oil ethanol was added to the supercritical CO_2. No significant increase was achieved in the yield of extract with the addition of such modifier. This result reveals the similarity in the solubility of the largest part of cardamom extract in SC-CO_2 and ethanol. Accordingly, the modifier needed to increase the yield should modify the solubility of less polar fraction of the oil extract. The recommended modifier is propane as discussed earlier.

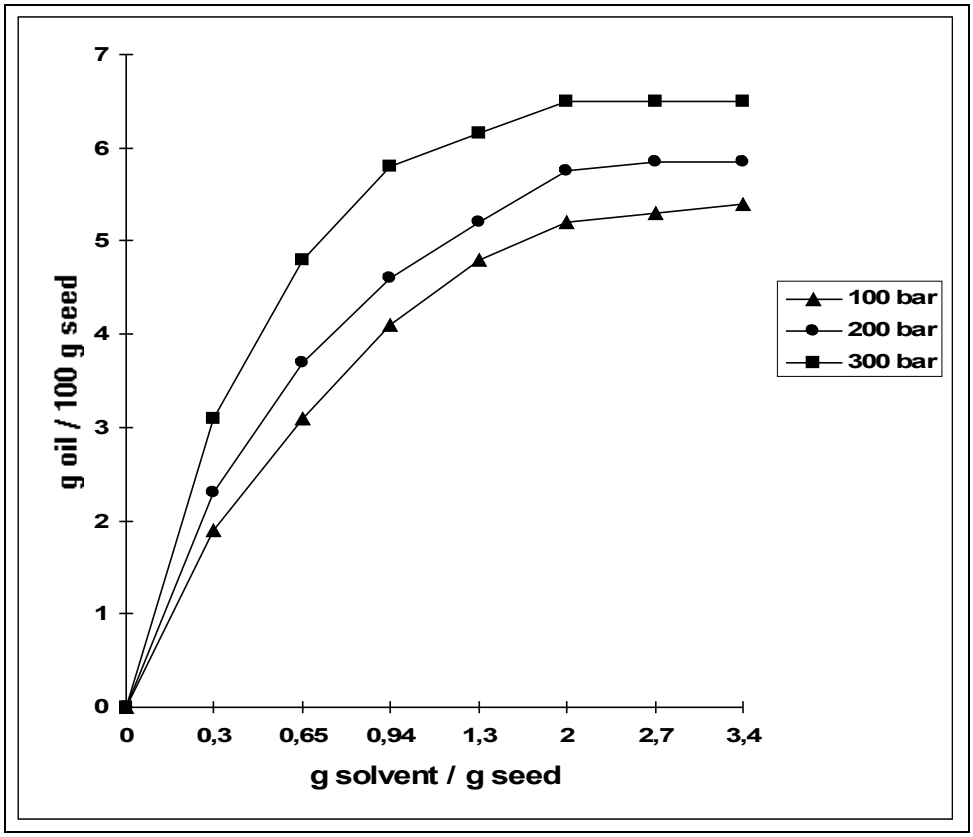


Figure 29. Supercritical CO_2 extraction of cardamom seeds as a function of the solvent/ solid ratio at different pressure values and 35°C.

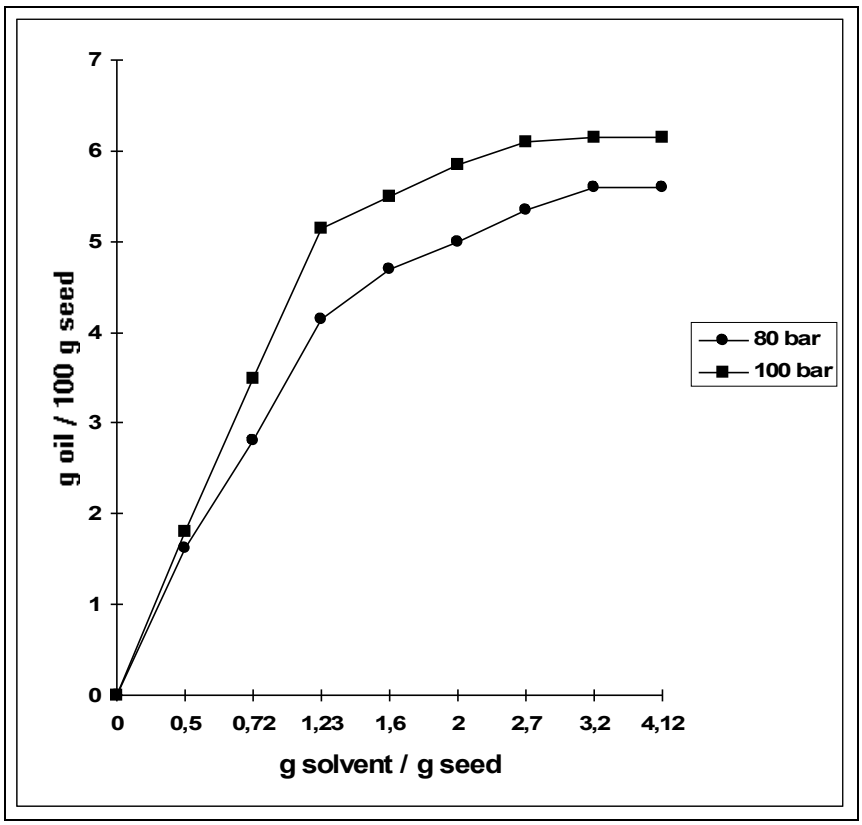


Figure 30. Sub-critical CO_2 extraction of cardamom seeds as a function of the slovent/ solid ratio at two pressure values and 25°C.

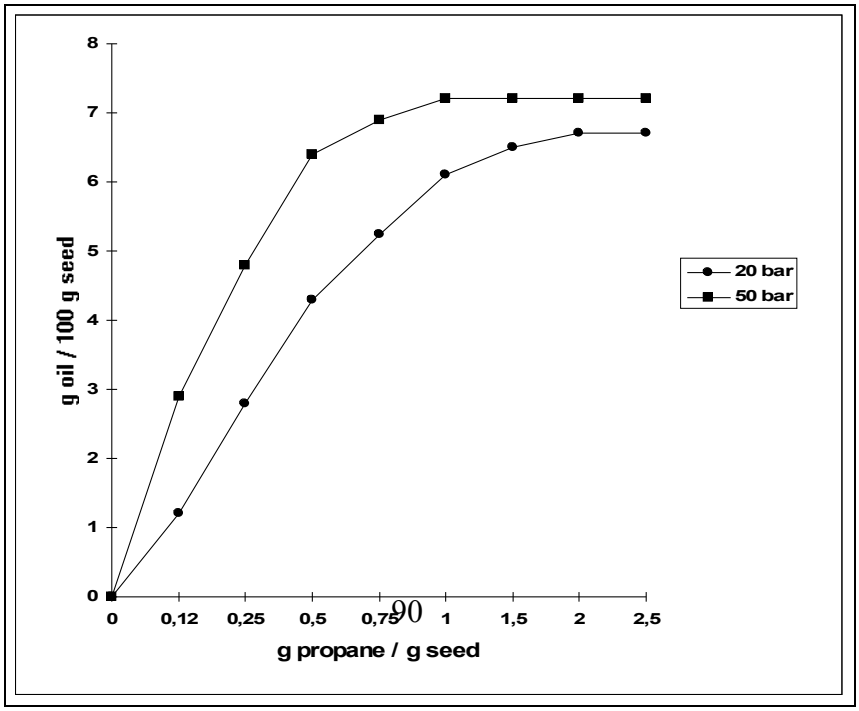


Figure 31. Sub-critical propane extraction of cardamom seeds as a function of the slovent/ solid ratio at two pressure values and 25°C

4.2.5. Effect of extraction on pigments, tocopherol, and volatiles

On pigments

Sub-and supercritical extraction conditions affected, to a considerable extent, the fat-soluble pigment content of the extracts. As shown in Table 16, solubility of fat-soluble pigments in SF- CO_2 is affected, to a considerable extent, by pressure. Recovery of carotenoids with SF-CO_2 increased proportionally to the increase of pressure. The β-carotene content of the extract increased from 0.8 to 5.8 μg/g when the pressure was raised from 80 to 300 bar, while a 6.9-time increase was recorded for the total chlorophyll content. At pressure lower than 300 bar the super- or sub-critical CO_2 was not capable to extract chlorophylls and pheophytins from the tissues of the seeds. It seems that the high pressure and use of less polar modifier is necessary to dissociate such pigments from the tissues of the outer shell of the seeds. The total amount of each pigment extracted from the seeds increased 2.1-3.2 times when sub-critical propane was applied as the solvent for the extraction of such product indicating that sub-critical propane improves not only the yield but also the content of vital phyto-chemicals in the extracts. The maximum level of chlorophylls and pheophytins was approached with sub-critical propane extraction at 50 bar and 25°C. The β-carotene content of sub-critical propane extracted samples was significantly higher ($p=0.001$) than that determined in SF-CO_2-extracted oils.

Performance of extraction with sub-critical-CO_2 containing ethanol as modifier improved, to a high extent, solubility of chlorophylls and pheophytins and, slightly, solubility of β-carotene. It is therefore recommended to use a mixture of CO_2 and ethanol for the extraction of chlorophyll-rich extracts under sub-critical conditions

Table 16: Effect of extraction conditions on the yield and pigment content of cardamom seed oil

Extraction conditions		Yield	β-carotene		Chlorophylls		Pheophytin	
Pressure Bar	Temperature °C	g extract/100g seeds	µg/g oil	Rec%	µg/g oil	Rec%	µg/g oil	Rec%
Extraction with CO_2								
300	35	6.65±0.41	5.8±0.44	39	4.53±0.27	38	2.36±0.12	51
200	35	5.95±0.40	3.9±0.23	26	0.36±0.03	3	0.33±0.02	71
100	35	5.45±0.37	2.1±0.17	14	0.30±0.01	2,5	Tr	
100	25	6.30±0.51	1.4±0.51	9.5	0.60±0.04	5	Tr	
80	25	5.65±0.34	0.8±0.34	5.4	0.65±0.04	5	Tr	
Extraction with propane								
50	25	7.24±0.37	18.6±1.12	125	10.80±0.87	90	4.40±0.31	95
20	25	6.85±0.41	16.2±1.14	109	3.40±0.21	28	2.10±0.14	45
Extraction with CO_2+ ethanol								
100	25	5.28±0.36	1.64±0.11	11	9.65±0.51	80	2.60±0.18	56
4. Solvent Extraction **		7.63±0.31	14.80±0.31		11.95±0.60		4.60±0.23	

The values are the averages of 2 replications ± standard deviation

Tr: traces

Rec % : Recovery percentage of each compound as related to raw material

*** Solvent extraction of raw material as a control*

From Table 16 we can see that recovery % of extraction by sub-critical propane was higher for β-carotene than by organic solvent. It can be said that for β-carotene extraction propane is more suitable specialy at 50 bar.

On tocopherols

As shown in Table 17, the content of tocopherols in the oleoresin increases proportionally to the increase of extraction pressure reaching a level of 117.7; 9.2 and 28.9 µg/ g oleoresin for α-β- and γ-tocopherol which is close to those values recorded for tocopherols in Soxhlet-extracted oil fraction. These results and those founded with thyme extraction confirm that high pressure levels are needed to achieve high solubility in SC-CO_2 and agree with the results found by *(Gnayfeed et al.,2001 and Daood et al., 2002)* for the extraction of tocopherol from spice paprika.

Ethanol when added to SC-CO_2, to improve yield and extractability of some effective constituents under sub-critical conditions, did not result in a substantial change in tocopherol content. Furthermore the tocopherol content of the extract is significantly lower (p=0,05) than that found in the extract delivered without ethanol applying the same SFE conditions. The low level of tocopherol in the extract with CO_2 + ethanol is most likely due to the dilution by ethanol that was not removed or evaporated from the extract.

The other attempt to produce cardamom oleoresin with better properties was to use propane under sub-critical conditions as recommended by several authors working on different natural products including spices and medicinal plants *(Illés et al., 1997; Gnayfeed et al., 2001; Daood et al., 2002).*

The data in Table 17 emphasize that sub-critical propane extraction resulted in the maximum or close to the maximum level of tocopherols in the oleoresins obtained by other methods such as SFE at high pressure or soxhlet using petroleum ether or n-hexane. Since sub-critical propane has an important advantage concerning high oleoresin yield and low solvent / solid ratio it is repeatedly recommended to be applied to produce oleoresin with outstanding properties from cardamom seeds. The other trial to improve yield and properties of cardamom oleoresin was the mixing of cardamom at 1/1 ratio with paprika seeds that have considerable amounts of tocopherols mainly γ-homologue, the chemically reactive component of vitamin E *(Biacs et al., 1992).*

Because paprika seed oil contain mainly neutral lipids (of low polarity) it was not necessary to carry out the extraction at low pressure (90-100 bar). Glyceride- rich plant oils require SFE to be performed at pressure over 300 bar for the achievement of economic recovery of plant oils (*Stahl et al., 1980).*

The HPLC analysis of tocopherol from the different extracts indicated that γ-tocopherol is the major component accounting for more than 90% of the total tocopherol homologues with α- and δ-isomers being the minor ones. SFE of paprika seeds at 450 bar gave an oleoresin having lower γ- and δ-tocopherol ($p= 0.05$), but higher α-tocopherol ($p=0,001$) content as compared to the content of these homologues in the extract recovered by soxhlet method using petroleum ether. These results confirm the sensitivity of the highly reactive γ-and δ-tocopherol (chemically reactive antioxidant) to high pressure processing under the conditions used, and susceptibility of α-tocopherol soxhlet extraction and complete evaporation of the organic solvent.

SFE of mixture of cardamom and spice paprika seeds at 450 bar and 40°C yielded oleoresins with high level of γ-and δ-tocopherols, but α-tocopherol content decreased from 117.7 to 21.7 μg/g as a function of dilution with paprika oil. The same tendency was observed for β-tocopherol and holds true with both SFE and Soxhlet extraction procedures.

Table 17: Changes in tocopherol content of oleoresins delivered from cardamom seeds alone or mixed with paprika seeds using different extraction methods.

Materials	Extraction conditions			Tocopherol content μg/g			
	Pressure bar	Temp. °C	Solvent	α-Toc.	β-Toc.	γ-Toc.	δ-Toc.
Cardamom seeds	100	35	CO_2	68.1±3.1	4.0±0.2	10.3±1.1	Tr
Cardamom seeds	100	25	CO_2 +ethanol	57.8±2.7	3.4±0.3	8.6±0.9	Tr
Cardamom seeds	300	35	CO_2	77.1±6.8	6.4±0.4	23.5±1.7	Tr
Cardamom seeds	450	35	CO_2	117.7±7.5	9.2±0.6	28.9±1.8	Tr
Cardamom seeds	50	25	Propane	124.5±9.2	9.8±0.6	29.6±2.3	Tr
Cardamom seeds	Soxhlet		Petr.ether	124,5±9.7	10.2±0.8	26.4±2.1	Tr
Cardamom+ paprika	300	35	CO_2	20.1±2.2	Tr	582.6±34.2	3.4±0.2
Cardamom+ paprika	450	35	CO_2	21.7±1.3	Tr	646.6±26.2	3.9±0.4
Cardamom+ paprika	Soxhlet		Petr.ether	26.2±1.7	Tr	750.3±45.1	7.6±0.5
Paprika seeds	450	35	CO_2	7.6±0.4	Tr	706.8±26.8	5.7±0.3
Paprika seeds	Soxhlet		Petr.ether	3.1±0.2	Tr	794.3±47.2	8.1±0.4

Tr = traces

On volatiles

Table 18 shows the composition and area% of the most important volatiles of cardamom extract as affected by supercritical extraction conditions. Since such qualitative analysis is conducted with high experimental error (standard deviation) it cannot be exploited to estimate the real changes took place as a function of extraction process. Nevertheless, some interesting compositional changes could be observed, for example the higher extraction pressure applied (450 bar at 35˚C) caused sabinene, 1,8 cineol, α-terpineol and 4-terpineol to substantially increase and neral to dramatically decrease. On the other hand, the extracts from mixture of cardamom and spice red pepper(paprika) seeds had the same composition of aroma compounds with slight increase in the % of 1,8 cineol, linalool, α-and 4-terpineol and marked a decrease in the % of the others most likely due to the dilution effect of paprika oil.

Table 18: Effect of extraction pressure on composition and content (peak area %) of volatile in oleoresins extracted from cardamom seeds alone or with paprika seed (extraction temperature was 40°C)

Compounds	% of peak area			
	Cardamom oleoresin, 300 bar	Cardamom oleoresin, 450 bar	Cardamom + paprika oleoresin, 300 bar	Cardamom + paprika oleoresin, 450 bar
α-thujene	0.2	0.3	0.2	0.2
α-pinene	1.1	1.5	0.6	0.6
Sabinene	3.4	4.1	2.7	2.5
Myrcene	1.7	2.0	1.3	1.3
1,8- cineol	26.7	32.4	39.5	34.7
γ-terpinene	0.4	0.5	0.4	0.3
γ-terpinene	0.3	0.4	0.3	0.3
α-terpinolene	0.4	0.4	0.3	0.3
Linalool	2.1	2.2	2.7	2.7
4-terpineol	0.7	1.0	0.7	0.6
α-terpineol	0.6	1.9	1.2	1.4
Terpinene	0.7	1.8	1.2	1.3
Neral	2.2	0.3	0.3	0.3
Linalyl-acetate	11.5	10.9	6.4	7.1
Geranial	0.5	0.5	0.5	0.6
α-terpinenyl acetate	44.4	37.8	40.9	44.5

To study the quantitative changes in terms of weight % of the major volatiles in the different extract, internal standard were used with extraction by diethy ether followed by direct injection on the GC-MS instrument as described in Materials and methods.The obtained results are illustrated in Table 19.

Table19: Content and composition of volatile compounds of different cardamom extracts as affected by extraction condition

Volatiles	Ret. Time	Content, weight % g/100 g						
		SFE, 100 bar	SFE, 300 bar	SFE, 450 bar	Propane 50 bar, 25 °C	CO_2+ethanol 100 bar, 25°C	Soxhlet	Cardamom seeds
Sabinene	8.37	0.7± 0.1	0.3±0.0	0.4±0.1	1.1± 0.1	0.3 ± 0.02	0.9±0.1	0.14±0.01
1,8-cineol	9.75	13.9± 0.2	8.3±0.7	8.6±0.1	15.8 ± 1.2	8.1 ± 0.5	13.2±0.3	1.49±0.06
α-terpineol	13.01	1.4±0.2	1.1±0.0	0.9±0.1	3.4 ± 0.6	0.4 ± 0.1	0.9±0.1	Nd
g-terpinene	13.44	0.8±0.0	0.4±0.1	0.3±0.0	Tr	Tr	0.5±0.1	Nd
Linalyl-acetate	13.99	4.8±0.4	3.2±0.0	2.8±0.1	2.9 ±0.4	0.2 ± 0.0	2.3±0.4	0.12±0.01
Terpinyl-acetate	15.58	55.1±3.7	39.2±0.9	35.0±1.9	21.4 ± 1.7	4.6 ± 0.3	26.0±1.5	1.68±0.06
Bizabolene	18.54	1.0±0.3	0.5±0.3	0.6±0.0	Tr	Tr	0.2±0.0	Nd
Total GC-MS		76.0±3	52.70.2	49.8±2.5	45.9 ± 3.8	14.7 ± 0.7	43.9±1.5	3.47±0.10
Total, GC FID		75.70	49.35	51.70	*	*	49.00	4.35

*= not determined
Tr = traces
Nd = under detectable limit

The weight % of most of the volatiles tended to significantly decrease when the pressure of SFE was rised over 100 bar indicating that extraction at sub-critical or close to sub-critical conditions are necessarily required to obtain oleoresin characteristic aroma profile from cardamom seeds.

The content of the major aroma compounds, 1,8-cineol, linalyl acetate and terpinyl acetate, was 1,62 ; 1,71 and 2,12 times respectivly higher in oleoresin extracted at 100 bar and 35˚C as compared to that delivered at 450 bar.

In comparison between SFE- and Soxhlet-extracted samples it was observed that except 1,8-cineol the content of all cardamom volatiles in Soxhlet-extracted oleoresin is significantly lower than that found in oleoresin extracted with SC-CO_2 at 100 bar and 35˚C, this reveales the higher stability of 1,8-cineol

and low stability of the others during evaporation of the organic solvent (petroleum ether) which is well known to initiate chemical alteration on the molecules of most of volatile compounds. It is of interest to mention that the ratio of 1,8-cineol / terpinyl acetate in the raw material (ground cardamom seeds) was 0,89 and shifted to around 0,25 and 0,51 in extracts delivered by SFE and soxhlet respectively. Such a change emphasizes that 1,8-cineol is more susceptible to the SFE condition than terpinyl acetate.

It is also important to mention that the sum of volatiles determined with GC-MS agrees with that recorded by using GC-FID (flame ionization detector).

Performance of extractions under sub-critical conditions using the less polar propane resulted in extracts with higher content of 1,8-cineol and α-terpineol and lower content terpinyl acetate as compared to that found in extracts produced with SC-CO_2 and petroleum ether (Soxhlet). If the other advantages of sub-critical propane-extraction such as color intensity, antioxidant content and yield, are considered in the evaluation of the oleoresins it can be said that propane extraction under sub-critical is a convenient way to produce cardamom oleoresin with outstanding properties.

To investigate the effect of convenient modifier on the extractability of aroma compounds in SC-CO_2, ethanol was mixed at a ratio of 25% with CO_2 before extraction. An alcoholic extract with low density, deep greenish brown color and light odor (not acceptable as cardamom extract). Although 1,8-cineol level was close to that found in oleoresins extracted by SC-CO_2 at high pressures the content of the characteristic volatile compound (α-terpinyl acetate) was dramatically decreased as a result of ethanol addition. The low level of aroma compound in extract delivered with ethanol is most likely due to negative effect of the modifier on solubility of some major volatiles in SC-CO_2 or / and to dilution by alcohol, which remains in the extract after evaporation of CO_2.

Alcohol containing cardamom extracts may have special application possibilities in the field of pharmaceutical and chemical industries, where aroma profile and intensity are not the main interest.

On fatty acids

Another quality attribute of cardamom oil is the fatty acid composition. As shown in Table 20, cardamom seeds distributed 17 different fatty acids. In addition, degraded products appeared on the GC-MS profile. Under certain SFE conditions there were remarkable changes in the fatty acid composition of the extracts. For example decreasing the pressure to 100 bar and 35°C caused the recovery of C18:1 and C18:2 acids to considerably decrease, while C14:0, C17:0 and unidentified fatty acid derivatives (most likely oxidized products) tended to increase in the extracts obtained under such extraction conditions. By lowering the temperature to the sub-critical range 25°C degradation of fatty

acids could be moderated. These results indicated that complete recovery of plant oils with extraction at 100 bar needs more than 3-6 h to be achieved. Oxidation of fatty acids is expected to be accelerated particularly at high temperatures, therefore, such extraction should be performed at lower temperatures. With sub-critical propane extraction the different parameters did not substantially altered the fatty acid composition of cardamom seed oil. The fatty composition of propane-extracted oils was close to that of oil delivered with SF-CO_2 at 200 bar and 35°C. The lower levels of degraded products of fatty acids in oils obtained by sub-critical propane extraction (short time extraction) supports the conception that long time of exposure during SFE at 100 bar is responsible for the oxidative degradation of fatty acids. These results also confirmed that addition of ethanol, as modifier, does not improve fatty acid extractability by SF-CO_2. The fatty acid composition of oil recovered with CO_2 + ethanol (25%) was very similar to that of oil extracted with sub-critical propane. From these results it seems that addition of ethanol can shorten the time of extraction and affect positively on the color quality of the extract as mentioned earlier.

Table 20: Fatty acid composition of cardamom oil as affected by extraction conditions

Fatty Acids	Extraction with CO_2				Extraction with Propane		CO_2+ ethanol
	100 Bar	200 Bar	80 Bar	100 Bar	20 Bar	50 Bar	100 Bar
	35°C	35°C	25°C	25°C	25°C	25°C	25°C
C12:0	2.93	0.78	1.74	1.43	0.94	1.24	1.18
Unidentified	9.25	2.54	5.95	4.77	2.90	3.31	3.40
C14:0	11.5	2.14	4.89	4.13	2.08	2.67	2.41
unidentified	3.81	1.50	1.99	0.68	0.22	0.24	0.27
C15:0	0.79	0.33	0.53	0.48	0.26	0.23	0.29
C16:0	21.9	26.7	24.8	26.8	24.9	25.4	25.6
C16:1	2.96	2.07	2.59	2.23	1.93	1.94	1.98
C17:0	2.58	0.86	1.19	1.07	0.72	0.77	0.72
C17:I	0.86	0.50	0.67	0.55	0.43	0.45	0.43
unidentified	4.79	0.37	1.29	1.00	0.14	0.24	0.15
CI 8:0	1.81	1.92	1.81	1.64	1.89	1.79	1.95
C18:1	26.1	43.6	36.0	39.6	43.3	42.0	42.1
C18:2	5.40	11.8	9.33	9.16	13.9	13.6	13.8
C18:3	6.70	2.48	1.60	1.51	3.47	3.52	3.81
C20:0	1.28	0.83	0.92	0.83	0.97	0.81	0.79
C20:1	1.39	0.60	1.14	0.97	0.77	0.60	0.52
C20:2	1.09	0.32	1.02	0.82	Tr	tr	Tr
Σ %	99.2	99.4	97.5	97.5	98.9	98.8	99.4

The values represent average of two replications of fatty acid analysis for one pooled oleoresin in each treatment

4.2.6. Antioxidant capacity of cardamom products

Cardamom and paprika seeds showed significantly higher antioxidant capacity than the oleoresins. In addition, cardamom seeds exhibit higher antioxidant activity ($p= 0.001$) than that of paprika seeds. Such a great difference is almost likely due to the higher content of antioxidant phenol compounds in cardamom seeds. The alcohol extract of cardamom was brown-coloured while alcohol extract of paprika was paleyellow-coloured.

There was a significant increase in antioxidant capacity of oleoresins by increasing extraction pressure. In case of cardamom and paprika oil, no significant difference was found in the maximum antioxidant capacity achieved at higher extraction pressure levels. But cardamom oleoresins obtained at low extraction pressure, values exhibited significantly lower antioxidant capacity as compared to that estimated for oleoresins from mixture of cardamom and paprika seeds. This indicates that paprika seeds contain antioxidant can be easily extracted by SC-CO_2 even at sub- critical conditions.

Antioxidant capacity of paprika seed oleoresins recovered by SFE was not effected by the applied pressure level. These results do not agree with the believe that antioxidant properties of paprika seeds are due to tocopherol content as the extracts of paprika seed obtained at different pressure contained different levels of tocopherol see Table 21.

To see how essential oils of cardamom contribute to the antioxidant activity of the product, water vapour distillation was performed and the essential oils fraction was examined for antioxidant activity. A value of 0, 21 m mole/ kg was obtained. This value is close to that found for cardamom extract recovered at low pressure 100 bar and much lower than those found for other extracts of the product. From these results, it can be concluded that: 1) at low pressure values the extract of cardamom consists of mainly essential oils. 2) The essential oil fraction contributes to about 16% of the maximum antioxidant activity of SF-extracted oleoresin.

Table 21: Antioxidant activity of cardamom and paprika seeds and their oleoresins prepared by different extraction method.

Samples	Extraction conditions	TEAC mmol/kg
Cardamom seeds	**SFE, 100 bar, 40 °C**	**0.1 6 ± 0.06**
Cardamom seeds	**SFE, 300 bar, 40 °C**	**1.13 ± 0.15**
Cardamom seeds	**SFE, 400 bar, 40 °C**	**1.35 ± 0.04**
Cardamom + paprika seeds	**SFE, 300 bar, 40 °C**	**0.88 ± 0.03**
Cardamom + paprika seeds	**SFE, 400 bar, 40 °C**	**1.29 ± 0.14**
Paprika seeds	**SFE, 400 bar, 40 °C**	**1.24 ± 0.03**
Paprika seeds	**Soxhlet, pet.ether**	**1.23 ± 0.16**
Cardamom seeds	**Soxhlet, pet.ether**	**1.13 ± 0.02**
Cardamom seeds	**Steam distillation**	**0.21 ± 0.02**
Paprika seeds	**MeOH DPPH**	**5.46 ± 0.15**
Cardamom seeds	**MeOH DPPH**	**9.67 ± 0.03**

These results lead to the conclusion that most of the effective antioxidants of both cardamom and paprika seeds are hardly extracted by SC-CO_2 or organic solvents remain in the residues after extraction. Also, it was evident that changing the conditions of SFE can partially improve solubility of some phenol antioxidant. In order to obtain oleoresins with high antioxidant capacity solubility of phenol compounds should be improved by using suitable modifiers with SC-CO_2.

The values obtained for antioxidant capacity of oleoresin from cardamom seeds are in the range found by *Tuberoso et al., 2007* for different oil seeds.

4.2.7. Antibacterial properties of cardamom and paprika seeds oleoresins prepared by different extraction methods

Cardamom oil is shown to have antibacterial and antifungal actions. Badei et al., (1991), studied the antibacterial properties of dried cardamom to asses the potential usefulness of cardamom oil as a food preservative. The antibacterial effect of the oil was tested against 9 bacteria strains, 1 fungus, and 1 yeast, the oil was 28, 9% as effective as phenol *(Badei et al., 1991).*

Table 22, shows the inhibition of extracted oil on the tested bacteria. It can be seen that in case of *Bacillus cereus*, it was the most sensitive to the oleoresins.

Cardamom extracted by vapour distillation was less effective on *Bacilllus cereus* just like cardamom oil extracted at 100 bar comparing with cardamom oils extracted at 300 bar and 450 bar and by soxhlet. This indicates that the antibacterial compounds do not refer to the essential oil fraction.

For paprika seeds, the oil extracted at 450 bar and by Soxhlet showed inhibition only with a dilution of 10 times, this can be explained by the diffusion difficulties of the active substance from un-diluted solution in the agar. The inhibition of cardamom-paprika mixture on bacteria was mainly related to the inhibition effect of cardamom. For *L.monocytogenes* cardamom oleoresins with and without dilution showed inhibition while extract from mixture of cardamom+ paprika seeds did not show any inhibition against this bacteria when diluted preparation were examined.

In addition, only cardamom oil showed inhibition on *St. aureus* as shown in Table 23.The cardamom oil extracted by vapour distillation had the weakest inhibition while the other cardamom oleoresins extracted by supercritical and soxhlet did not show any significant difference in their inhibition against such microbe.

Also, cardamom oil only and just without dilution showed inhibition on *E.coli*. This indicates that the active substance against *E.coli* exists at low concentration in the extract. In case of *Pseudomonas aeruginosa* , none of the oils showed inhibition on it. So it can be concluded that only cardamom oil inhibited the tested bacteria and increasing the extraction pressure from 100 to 300 bar increased its inhibition effect. At 450 bar no significant change was seen. Soxhlet was the most suitable extraction method for antibacterial properties. Mixing cardamom with paprika decreases its inhibition. Figures showing the inhibitory effect of the extracts are shown in the appendix (Figures 33, 34, 35).

Table 22: Antibacterial properties of cardamom and paprika seeds oleoresins prepared by SFE at 40°C and different pressures, Soxhlet and vapour distillation

Sample		Extraction	Test Bacteria (inhibition zone (mm)				
			B. cereus	*L. monocyt.*	*St. aureus*	*Ps. Aerugino*	*E. coli*
Cardamom	Cardamom	100 bar	8.2	5.7	11.6	0	8.5
	10x dilution		8.8	6.2	8.9	0	0
	Cardamom	300 bar	15.0	8.1	8.3	0	8.5
	10x dilution		15.0	7.0	9.5	0	0
	Cardamom	450 bar	16.2	8.5	11.0	0	0
	10x dilution		15.5	7.3	9.0	0	0
	Cardamom	Soxhlet	15.1	15.2	11.5	0	10.0
	10x dilution		15.0	6.0	6.0	0	0
	Cardamom	Vapour disst	5.2	5.1	9.3	0	7.6
	10x dilution		5.7	5.1	7.0	0	0
Paprika	Paprika	450bar	0	0	0	0	0
	10x dilution		6.9	0	0	0	0
	Paprika	Soxhlet	0	0	0	0	0
	10x dilution		8.1	0	0	0	0
Cardamom + paprika	Card+paprika	300 bar	14.3	10.5	6.0	0	0
	10x dilution		8.8	0	0	0	0
	Card+paprika	450 bar	13.4	0	7.6	0	0
	10x dilution		8.0	0	5.1	0	0
DMSO			0	0	0	0	0
Inhibitors			11.6	33.7	33.9	0	13.6

4.2.8. Antifungal properties of cardamom and paprika seeds oleoresins prepared by different extraction method

As shown in Table 23, it can be seen that only non diluted cardamom oil showed inhibition on *Aspergillus niger* while parika and mixture did not show any inhibition. This means that the active substance against *Aspergillus niger* is present in low concentration in the cardamom extract.

Table 23: Antifungal properties of cardamom and paprika seeds oleoresins prepared by SFE at (40°C and different pressures), Soxhlet and vapour distillation

Sample	*Extraction*	*Aspergillus niger*	*P. vermiculatum*
Cardamom	**SFE 100 bar**	+	+
Cardamom 10x dilution	**SFE 100 bar**	-	+
Cardamom	**SFE 300 bar**	+	+
Cardamom 10x dilution	**SFE 300 bar**	-	+
DMSO		-	+
K-sorbate		+	+
Cardamom	**SFE 450 bar**	+	+
Cardamom10x dilution	**SFE 450 bar**	-	+
Cardamom	**Soxhlet**	+	+
Cardamom10x dilution	**Soxhlet**	-	+
DMSO		-	+
K-sorbate		+	+
Cardamom	**vapor distillation**	+	+
Cardamom10x dilution	**vapor distillation**	-	+
Paprika seeds	**SFE 450 bar**	-	-
Paprika seeds10x dilution	**SFE 450 bar**	-	-
DMSO		-	-
K-sorbate		+	+
Paprika seeds	**Soxhlet**	-	-
Paprika seeds10x dilution	**Soxhlet**	-	-
Cardamom +paprika	**SFE 300bar**	+	-
njdilution	**SFE 300bar**	-	-
DMSO		-	-
K-sorbate		+	+
Cardamom +paprika	**SFE 450bar**	-	-
Cardamom +paprika10x dilution	**SFE 450bar**	-	-
DMSO		-	-
K-sorbate		+	+

Also, cardamom extracted by all extraction methods and at all extraction conditions inhibited *Penicillium vermiculatum*. The mixture with paprika did not inhibit the mould. It indicates that the active substance against *Penicillium vermiculatum* is found in cardamom. Figures showing the inhibitory effect of the extracts are shown in the appendix (Figures 36, 37).

4.2.9. Antiyeast properties of cardamom and paprika seeds oleoresins prepared by different extraction method

The *K. lodderare* propagation was inhibited only by cardamom oil. The diluted samples were more effective on *K.lodderare* propagation. It means that the active substance which is responsible for inhibition can diffuse easier when diluted, see Table 24.

Table 24: Antiyeast (K.lodderare) properties of cardamom and paprika seeds oleoresins prepared by SFE at 40°C and different pressures, soxhlet and vapour distillation. (Inhibition zone,mm).

Samlpe	Extraction conditions	Non-diluted	10x dilution
Cardamom	**SFE 100 bar 40°C**	**10.0**	**9.2**
Cardamom	**SFE 300 bar 40°C**	**7.1**	**8.1**
Cardamom	**SFE 450 bar 40°C**	**8.1**	**9.6**
Cardamom	**Soxhlet**	**7.1**	**8.9**
Cardamom	**Vapour distillation**	**0**	**6.7**
Paprika	**SFE 300 bar 40°C**	**0**	**0**
Paprika	**Soxhlet**	**0**	**0**
Cardamom+paprika	**SFE 300 bar 40°C**	**0**	**0**
Cardamom+paprika	**SFE 450 bar 40°C**	**0**	**0**
DMSO		**0**	**0**
Potassium-sorbate		**0**	**0**

It can be concluded that only cardamom oil showed inhibition on the tested bacteria. Soxhlet was the most effective extraction method for cardamom. Cardamom oil extracted by SFE- CO_2 also showed inhibition on the tested bacteria, but no significant inhibition difference was seen when changing SFE-CO_2 conditions. Figure showing the inhibitory effect of the extracts is shown in the appendix (Figure 38).

5. SUMMARY AND NEW SCIENTIFIC FINDINGS

5.1. Summary:

1. HPLC analysis showed that thyme leaves are rich in vital carotenoids such as lutein and β-carotene which comprised up to 2850 and 650 μg/g dry weight in freshly harvested and artificially dried product respectively. As concerns the chlorophyll content in the leaves distributed approximately 10 and 3,3 mg/ g dry weight total chlorophylls in fresh and dried state respectively. With remarkably high level of oxidised chlorophylls even in the freshly harvested leaves indicates a high level of biochemical oxidation in such a crop. The tocopherol content was 650 and 195 μg/g dry weight in fresh and dried leaves respectively.
2. The leaves of dried thyme (raw material) contained mainly carvacrol, thymol, γ-terpinene and ρ-cymene as the major volatiles responsible for the characteristic aroma profile of the product. The high level of carvacrol, which accounted for 58,2% of the total peak area emphasized that the examined thyme belonngs to the wild species of thyme.
3. With SFE using CO_2 the yield of thyme oleoresin increased from 3,4 to 5,2 g/ 100g dried thyme when the pressure was raised from 100 to 400 bar, while extraction with propane at sub-critical condition yielded about 5,8 g oleoresin from 100g thyme with ratio of solvent/ solid 3 times than that found with SC-CO_2.
4. The SC-CO_2 was found to hardly solubilize fat-soluble pigments particularly chlorophylls. However, increasing the extraction pressure caused a slight improvement in the solubility of pigment at SC-CO_2. Solubility of carotenoid type pigments in SC-CO_2 at high pressure was higher than that of chlorophylls, but the highest recovery did not exceed 82% of the initial content in raw materials.
5. Propane at cost-effective conditions solubilized effeciently both chlorophylls and carotenoids. This type of extraction produced oleoresin with high tocopherol content.
6. With both SC-CO_2 and sub-critical propane carotenoids underwent all-trans to cis isomerization, which was pressure-independent.
7. Performing of SFE with CO_2 at 300 bar was the best for the extraction of carvacrol, ρ-cymene and β-myrcene whereas thymol, β-carophyllen and γ-terpinene showed decreasing tendency when the extraction pressure was changed from 100 to 300 bar.

8. Radical scavenging activity of thyme oleoresin increased significantly with increasing of the extraction pressure to 300 bar, but no significant change was found at high pressure levels.
9. Thyme extract obtained with different extraction SFE pressures show high inhibitory effect on the bacteria tested, even at 32 times dilution. All the pathogenic bacteria (*B. cereus , L. monocytogenes*, *St. aureus*, and *E. coli*) were more sensitive, than *Ps. aeruginosa*. In case of *Ps.aeruginosa* the best inhibitory effect was found with oleoresin delivered at 300 bar.
10. The recent analytical method used showed that cardamom seed contain lutein and β-carotene in addition to chlorophyll a and b and their derivatives as the naturally occurring pigments. The seeds were found to contain low level of vitamin E components such as β-and γ-tocopherol.
11. GC-MS analysis showed that terpinyl acetate, 1,8-cineol and linalyl acetate are the major constituents of essential oil of cardamom seeds. These compound comprised up to 82,6% of the total aroma profile in the oleoresin of this crop.
12. Extraction with SC-CO_2 at 300 bar gave maximum oleoresin yield of 6, 65 g from 100 g ground seeds of cardamom. The yield of oleoresin was increased to 7, 24 when propane at sub-critical condition was applied.
13. The content of fat-soluble pigments in the extracts were increased by increasing the extraction pressure up to 300 bar, e. g β-carotene level increased from 0,8 to 5,8μg/g oleoresin. The extract produced under different SFE conditions contained pheophytins as fat-soluble chlorophyll derivative. Performance of extraction with propane at sub-critical condition improved the content of the chlorophyll, and carotenoids in the extracts. Use of ethanol as modifier improved the solubility of chlorophylls and carotenoids in SC-CO_2 at sub-critical conditions.
14. The tocopherol content of cardamom oleoresin increased proportionally to the increase of extraction pressure with SC-CO_2. The maximum tocopherol content achieved with SC-CO_2 was very close to that estimated in extracts obtained by sub-critical propane or organic solvent (soxhlet). Extraction of mixture of cardamom and paprika seeds resulted in an oleoresin having high level of α- and γ- -tocopherols, the chemically reactive fat-soluble antioxidants.
15. The highest content of the major volatiles of cardamom was found in oleoresin prepared by SC-CO_2 at 100 bar (near sub-critical conditions). These conditions are suitable for extraction of essential oils from cardamom. Performance of SFE at higher pressure levels gave oleoresins with low content of the characterestics aroma compounds. Using of ethanol as a modifier to SC-CO_2 did not improve solubility and recovery of the essential oils.

16. Performance of extractions under sub-critical conditions using the less polar propane resulted in extracts with higher content of 1,8-cineol and α-terpineol and lower content terpinyl acetate as compared to that found in extracts produced with SC-CO_2 and petroleum ether (Soxhlet).
17. Under certain SFE conditions there were remarkable changes in the fatty acid composition of the extracts. For example decreasing the pressure to 100 bar and 35°C caused the recovery of C18:1 and C18:2 acids to considerably decrease, while C14:0, C17:0 and unidentified fatty acid derivatives (most likely oxidized products) tended to increase in the extracts obtained under such extraction conditions.
18. The lowest radical scavenging activity of cardamom extracts was found in that prepared at 100 bar and 40°C where essential oils comprised a great part of the extract. Similar antioxidant capacity was recorded for the extract prepared by water-vapour distillation. Improvement of tocopherol content by extract in cardamom and paprika seeds gave no significant improvement to the antioxidant activity of the extract. Application of high extraction pressure with SC-CO_2 caused the antioxidant capacity to substantially increase most probably due to solubilisation of some phenols having high antioxidant activity.
19. The different extract of cardamom exhibited antimicrobial activity toward all microbes examined except *Ps. Aeruginosa*, which was not inhibited by the active substance of cardamom. Non diluted cardamom oil showed inhibition on *Aspergillus niger* while parika alone or with cardamom did not show any inhibition. This means that the active substance against *Aspergillus niger* is in ineffective concentration in these extracts. In case of *Penicillium vermiculatum*, diluted and non diluted cardamom extracts showed inhibitory effect on the growth of this mould. Addition of paprika seeds decreased effectiveness of cardamom extract against the mould growth. The same inhibitory effect of cardamom extract was found on the yeast *K.Iodderare*.

5. 2. New Scientific Findings

The new scientific findings derived from the research work are summarised as follows:

1. Super- and sub-critical carbon dioxide hardly solubilises the chlorophyll type pigments and their derivatives from the tissues of chloroplast of dried thyme and cardamom seeds. The large part the pigment remains in the residues. As concerns, the carotenoid type pigments solubility in SC-CO_2 could be improved by rising the pressure of the SFE to 400 bar. However maximum recovery of 82% could be achieved with marked increase in the content of the cis isomers of

the major carotenoids such as lutein and β-carotene. Using ethanol as modifier with SC-CO_2 or application of less polar solvent such as propane produces oleoresins with high level of both chlorophylls and carotenoids.

2. Extraction of both thyme and cardamom with propane at sub-critical conditions yielded oleoresins having improved composition with special regards to the content of colourants, antioxidants and volatile compounds. Basing on the ratio of solvent/solid used for the approach of complete extraction of oleoresin sub-critical propane is a better solvent than SC-CO_2.
3. Extraction of thyme and cardamom seeds by SC-CO_2 or sub-critical propane can substantially alter the content and composition of naturally occurring pigments via enhancement of oxidation, isomerisation and hydrolysis of chlorophyll a and b. The extent of artifact formation as a function of super and sub-critical extraction processes seemed to be associated with the composition and chemical properties of the raw materials.
4. Thyme oleoresins having high antioxidant content and radical scavenging activity could be produced by extraction with SC-CO_2 at high pressures, which increased the tocopherol content and colour intensity (visually) of the extracts.
5. In case of cardamom, the raw materials and the obtained oleoresins were found to contain low level of the fat-soluble antioxidant (tocopherols) and relatively low antioxidant activity as well. Extraction of a a mixture of paprika and cardamom seeds to increase tocopherol content to a considerable level did not result in a significant improvement on the radical scavenging capacity of the extracts indicating that the antioxidant capacity of cardamom seeds is associated to high antioxidant activity of other constituents (most likely phenols).
6. It was found that radical scavenging activity in extracts rich in essential oils is significantly lower than that recorded for the other oleoresins revealing that essential oils of both thyme and cardamom seeds contribute only partially to the overall antioxidant activity of the products.
7. The major constituents of the essential oil part of both thyme and cardamom extracts were found to be negatively influenced by increasing the pressure of SFE. The greatest proportion of the volatiles of both herbs can be recovered in the extract when the extraction is performed at sub-critical conditions using either CO_2 or propane.
8. Oleoresins from both cardamom seeds and thyme prepared by different extraction methods had marked anti-microbial activity with thyme extracts being several time more effective than those of cardamom. The extract of the two herbs were found effective against different bacteria, yeasts and moulds.

9. Sub-critical propane was found more favourable for β-carotene extraction than other solvents.

6.References

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APPENDIX

I. Abbreviations and notation

ρ	density (kg/m^3);
ρ_C	critical density (kg/m^3);
λ	thermal conductivity (W/mK);
η	viscosity (μP);
η_C	critical viscosity (μP);
η_R	reduced viscosity (μP);
a	attraction parameter
ANOVA	analysis of variances;
B	van der waals co-volume;
BC	before christ;
BHA	butylated hydroxyanisole;
BHI	brain heart infusion;
BHT	butylated hydroxytoluene;
C	molar concentration (mol/mol);
Cfu	colony forming unit;
D	diffusivity (m^2/s);
DMSO	dimethy-sulphoxide;
DPPH	1,1-diphenyl-2-picrylhydrazyl;
FAMEs	fatty acid methyl esters standard
G	gas-meter;
GC	gas chromatography;
GC-MS	gas chromatography-mass spectroscopy;
H	cooled vessel;
HPLC	high-performance liquid chromatography;
IS	internal standard;
J	diffusion flux;
K_T	isothermal compressibility;
LOD	limit of detection;
LOQ	limit of quantification;
M	molecular weight;
Me	extraction column;
Msz	membrane pump;
Nd	not detected;
P	pressure (bar);

p_C	critical pressure (bar);
PDMS	polydimethyl siloxan;
PE	buffer vessel;
q	heat flux;
R	gas constant;
R	rotameter;
$scCO_2$	supercritical carbon dioxide;
SCF	supercritical fluid;
SD	standard deviation;
SPME	solid phase micro extraction;
SFE	supercritical fluid extraction;
T	temperature (°C);
TBHQ	tert-butylhydroquinone;
T_C	critical temperature (°C);
TEAC	Trolox equivalent antioxidant activity;
TE	thermostat;
Tr	traces;
Udl	under detection limit;
Z	direction.

II. Antimicrobial Tests of cardamom and paprika

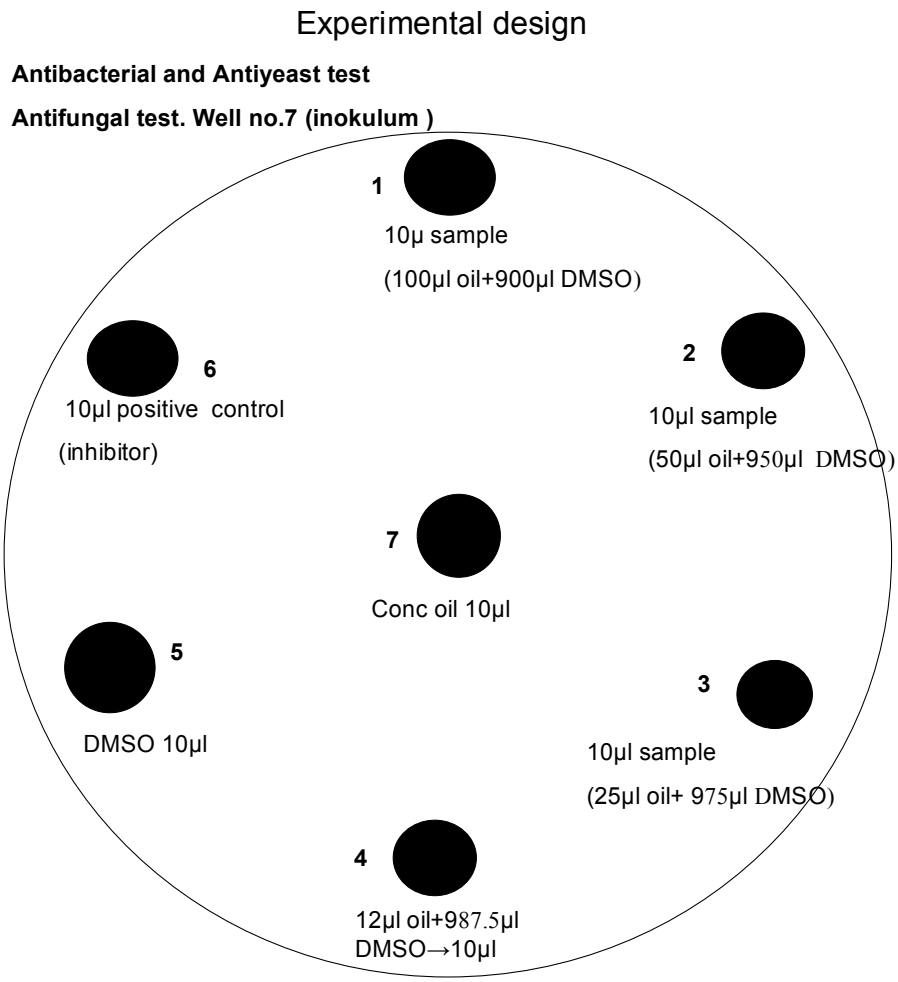
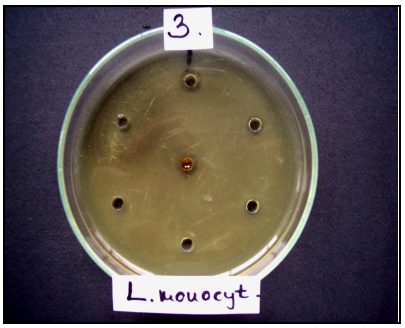
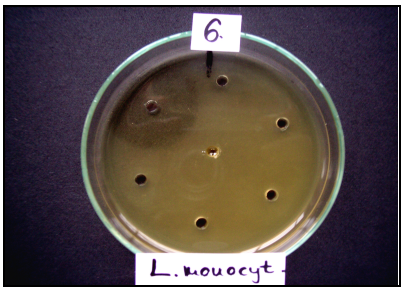


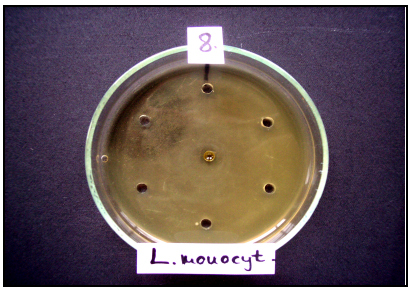
Figure 32. Experimental design for antimicrobial test

Listeria monocytogenes

Possitive control. *Ampicilin*

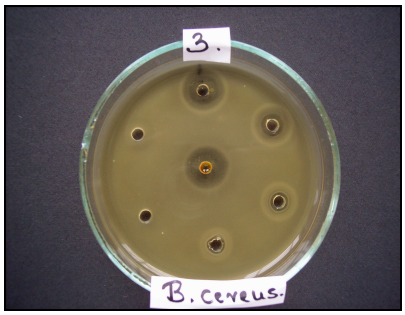


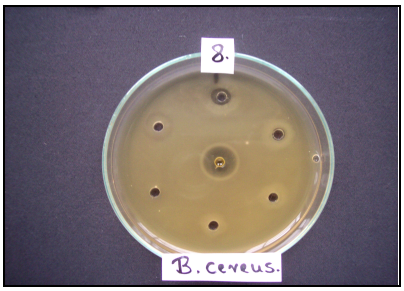




Bacillus cereus

Possitive control. *Ampicilin*





3-cardamom

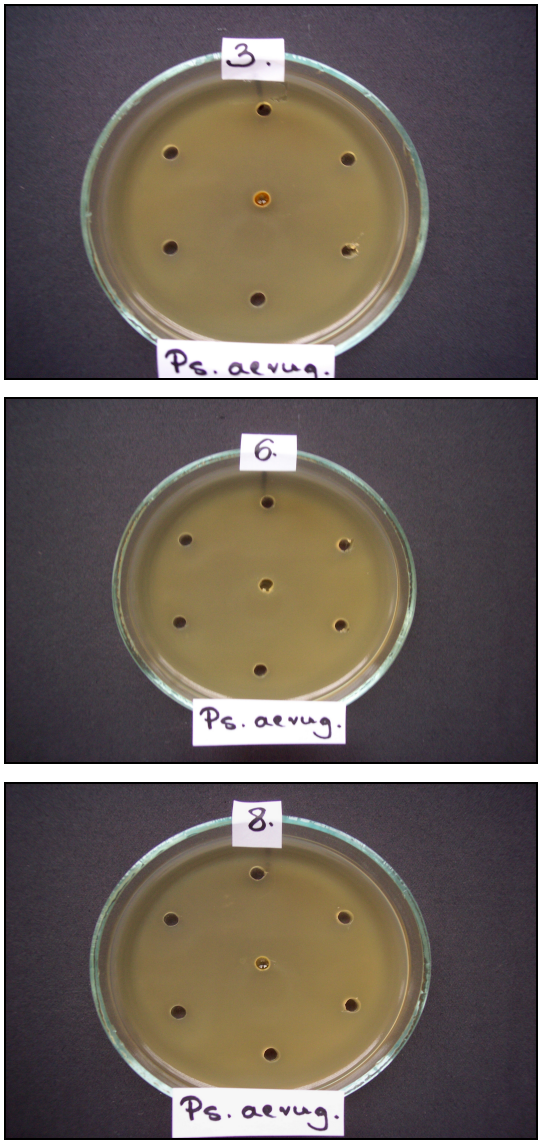
6- paprika

9- cardamom+ paprika

Figure 33. Antibacterial test of cardamom and paprika oils on Listeria monocytogenes and Bacillus cereus

Pseudomonas aeruginosa

Possitive control . *Ampicilin*



3-cardamom oil

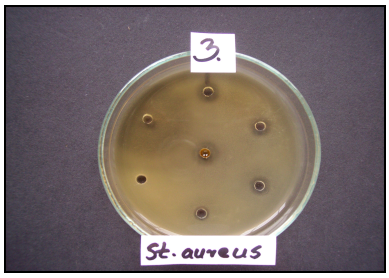
6- paprika oil

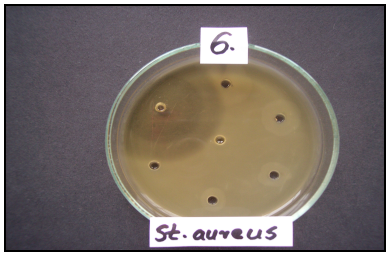
9- cardamom+ paprika oil

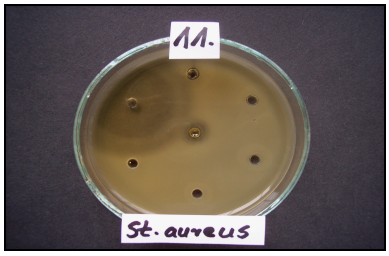
Figure 34. Antibacterial test of cardamom and paprika oils on Pseudomonas aeruginosa

Staphylococcus aureus

Positive control: *Cefalosporin*

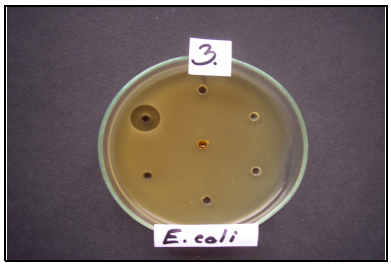


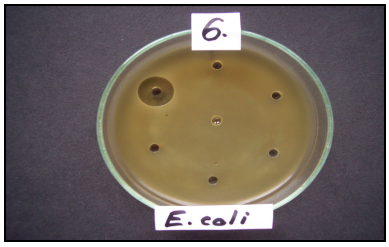


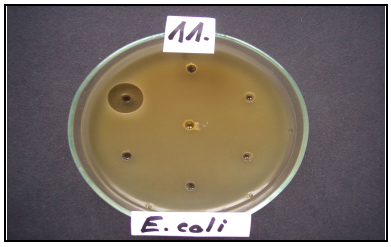


Escherichia coli

Positive control*: Polymixin*







3-cardamom oil

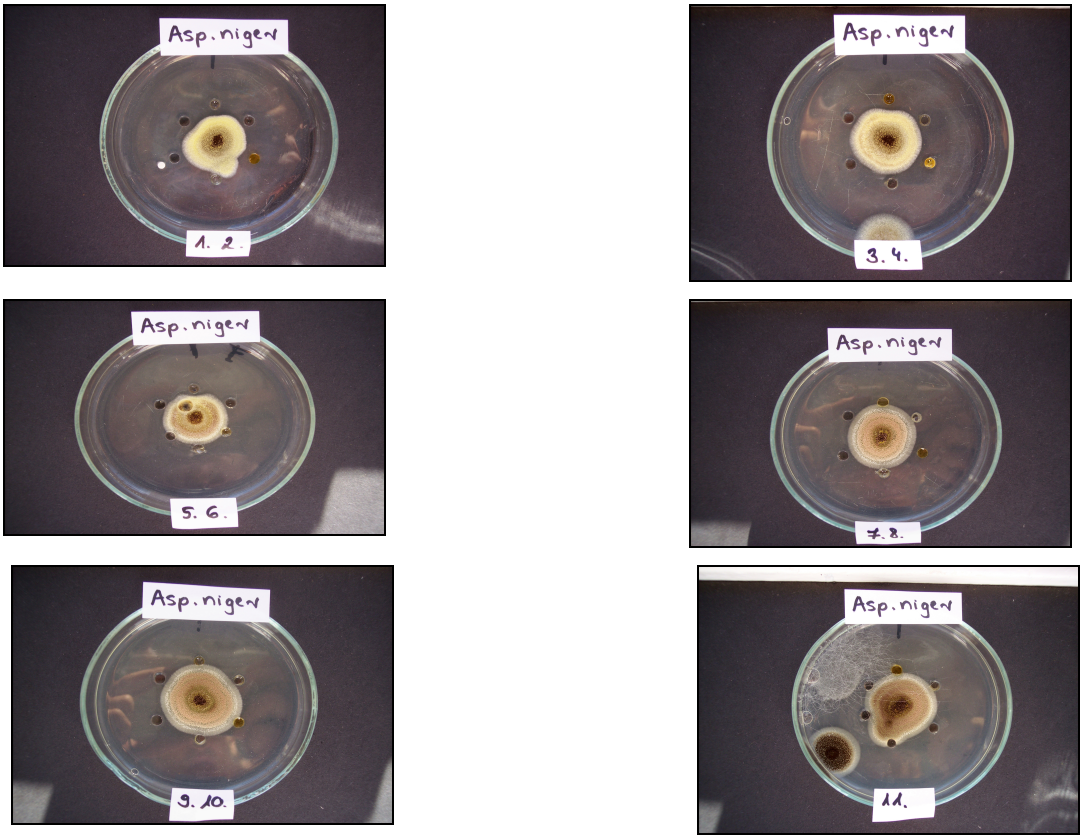
6- paprika oil

9- cardamom+ paprika oil

Figure 35. Antibacterial test of cardamom and paprika oils on Staphylococcus aureus and Escherichia coli

Aspergillus niger

Wells no.1: Conc., 2. 10x dilution, 3. Conc. Oil, 4. 10x dilution 5. Well DMSO, 6. Well: K-sorbate



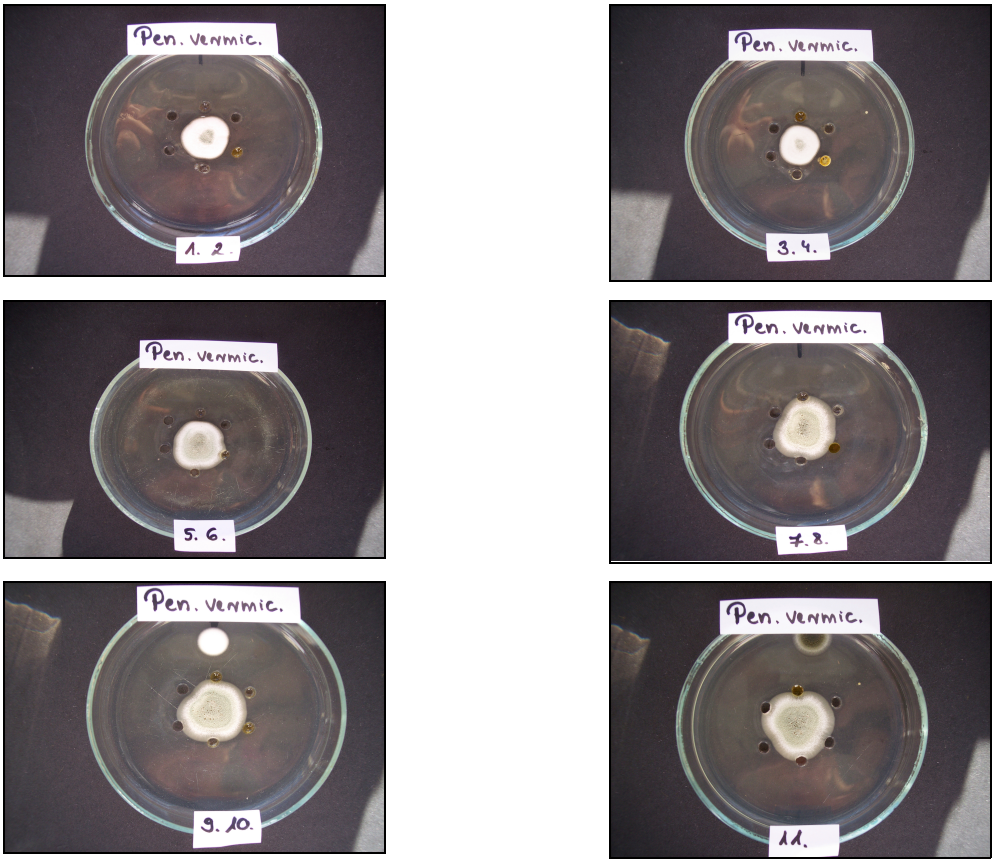
1-5: cardamom; 6-7: paprika, 8-11: cardamaom+paprika

For sample11 wells no. 4 and 6 are empty. 5. K-sorbate, 3. DMSO

Figure 36. Antifungal test of cardamom and paprika oils on Aspergillus niger

Penicillium vermiculatum

Wells no.1: Conc., 2. 10x dilution, 3. Conc. Oil, 4. 10x dilution 5. Well DMSO, 6. Well: K-sorbate

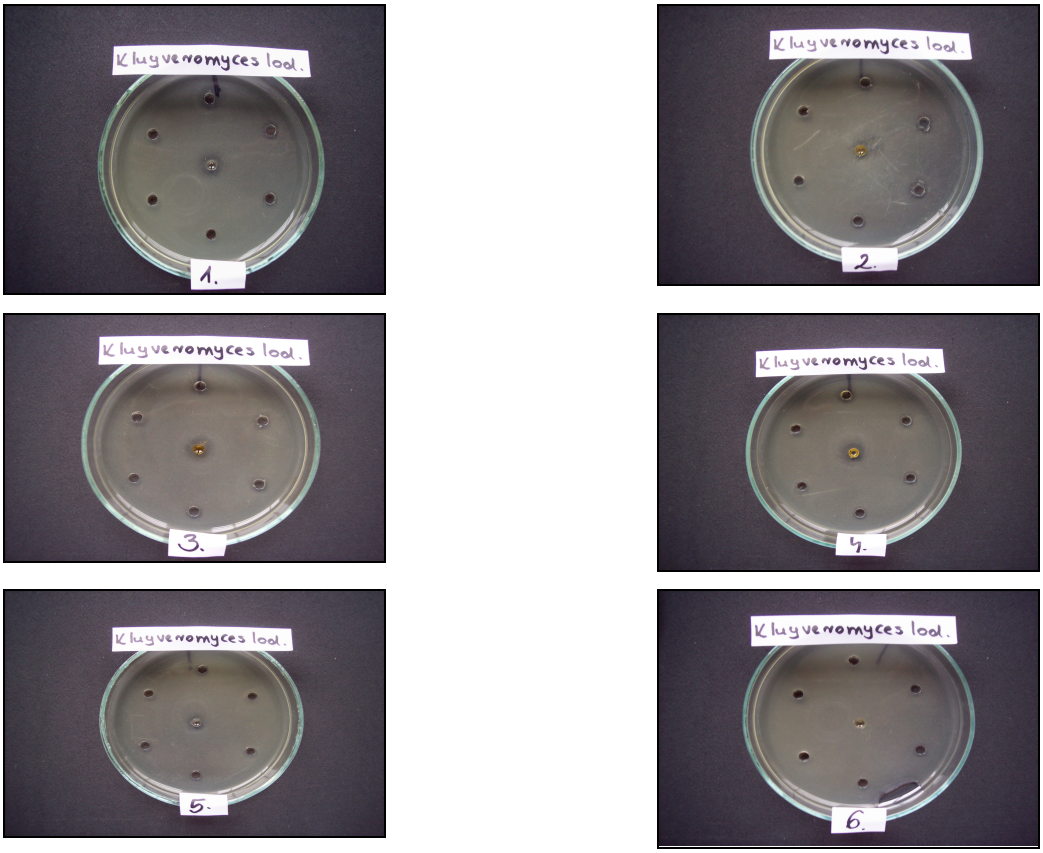


1-5: cardamom; 6-7: paprika, 8-11: cardamaom+paprika

For sample 11 wells no. 4 and 6 are empty. 5. K-sorbate, 3. DMSO

Figure 37. Antifungal test of cardamom and paprika oils on Penicillium vermiculatum

Kluyveromyces lodderare



1-5: cardamom oil, 6: paprikaoil

Figure 38. Antiyeast test of cardamom and paprika oils on Kluyveromyces lodderare

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