**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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## Introduction

There have been great efforts to prepare safe and potent extract from natural products that have nutritional, industrial and therapeutic 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. 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 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 effective materials with Special focus on the natural pigments, antioxidants and compounds in the raw material and extract.
4. Studying the changes in the antioxidant content and activity of thyme and cardamom extract as a function of SFE conditions.
5. Investigation of anti-microbial activity of thyme and cardamom extract produced under different conditions.

## Materials and Methods

### *Super and sub-critical extraction*

Forty-five grams of the freshly ground cardamom and thyme seeds were packed in the extractor of a high-pressure, flow-up stream extraction apparatus and $CO_2$ or propane was pumped up to the required pressure using a membrane pump. The solvent passes through a buffer vessel into the thermostat-controlled column. A back-pressure valve regulator adjusted the pressure in the extractor. Thermocouple-based indicating system and pressure transducer measured the temperature and pressure respectively. 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 $CO_2$ was determined by applying Peng-Robinson equation after volume measurement of the gas coming from the gas cylinder. 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. SFE studies were conducted to extract ground thyme and cardamom seeds with SF-$CO_2$ at 25°C-55°C and pressure of 80-450 bars. In the case of sub-critical propane, the extraction was carried out at 25°C and pressure of 20-50 bar.

**Chemical analysis**

***Analysis of carotenoids and chlorophylls***

To analyse carotenoids and chlorophylls HPLC was applied using reversed-phase C18 column with gradient elution starting with water containing methanol and ending with a mixture of acetonitrile-isopropanol-methanol. Detection was carried out b photodiode –array detector between 190-700nm. The compounds were identified by comparing their retention behavior and spectral characteristics with those of pure and authentic materials.

**Analysis of tocopherols**

To analyse tocopherol normal-phase HPLC was performed on Nucleosile column eluted with 99.6:0.4 n-hexane-absolute alcohol. The peaks were detected at Ex: 295nm and Em: 320nm. The homoluges of tocopherol was identified and quantificated by standard materials.

**Analysis of fatty acids**

The fatty acid composition of the extracts was analyzed by gas chromatography (GC). Before analysis the glycerin-esters of triglyceride were converted into methyl-esters according to the Hungarian Standard methods: No-19929-86 and No-195228-86.

Fatty acid composition was determined according to *Tóth and Sass-Kiss, (1993)* using borosilicate coated with supelcowax film as the stationary phase and $N_2$ as the carrier gas. Detection was performed with Flame ionization detector (FID). The temperatures program used was 180 °C-4°C/min up to 220 °C final temperature.

**Analysis of aroma compounds**

To analyse aroma compounds GC-MS was used. GC-MS under the following conditions:

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 in 5 minutes at 10 °C/min.

Injection: Split-less injection at 260 °C (PARR method).

Identification: According to a Wiley 275 library T data.

**Antimicrobial activity**

Tow gram-negative bacteria, (*Escherichia Coli and Pseudomonas aeruginosa*), three gram-positive bacteria, (*Bacillus cereus, Listeria monocytogenes and Staphylococcus*), two fungi, (*Penicillium vermiculatum and Aspergillus niger* ) and one yeast (*Kluyveromyces Ioddeare)* were used in the investigation of antimicrobial activity of oleoresins using agar diffusion method.

**Determination of antioxidant capacity**

Trolox equivalent antioxidant activity (TEAC) was used with DPPH radical in ethyl acetate to determine the radical scavenging activity of oleoresins. In case of raw materials the antioxidant capacity of filtrates was determined according using a procedure, in which 80% methanol solution was used for the DPPH reaction.

**Results**

The results achieved in this work could be summarised as follows:

1. HPLC analysis showed that thyme leaves are rich in vital carotenoids such as lutein and β-carotene which comprised upto 2850 and 650 μg/g dry weigh 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 resposible 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 is due 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$SC\text{-}CO_2$.
4. The SC-CO2 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 $SC\text{-}CO_2$. Solubility of carotenoid type pigments in $SC\text{-}CO_2$ 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 solubilized effeciently both chlorophylls and carotenoids. This type of extraction produced oleoresin with high tocopherol content.
6. With both $SC\text{-}CO_2$ 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 ( *B. cereus , L. monocytogenes, St. aureus, and E. coli)* even at 32 times dilution. All the pathogenic microbes were more sensitive to the extracts, than *Ps.aeruginosa* . In case of *Ps.aeruginosa* the best inhibitory effect was found with oleoresin delivered at 300 bars.
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 bars 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 bars, 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-CO2. The maximum tocopherol content achieved with SC-CO2 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 γ- and σ-tocopherols, the chemically reactive fat-soluble anzioxidants.
15. The highest content of the major volatiles of cardamom was found in oleoresin prepared by SC-CO2 at 100 bars (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-CO2 did not improve solubality 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-CO2 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 bars 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-CO2 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. *Penicillium vermiculatum* seemed to be more sensitive to the cardamom extracts, than *Aspergillus niger*, while paprika extract was ineffective against both moulds. Non diluted cardamom oil showed inhibition on *Aspergillus niger* while parika alone or with cardamom did not show any inhibition. 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 effectivness of cardamom extract against the mould growth. The same inhibitory effect of cardamom extract was found on the yeast *K.lodderare.*

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 larg part the pigment remains in the residues.As concerns the carotenoid type pigments their solubility in SC-CO2 could be improved by rising the pressure of the SFE to 400 bars. However maximum recoversy of ..% could be achieved with marked increase in the content of the cis isomers of the major carotenoids suvh as lutein and β-carotene. Using ethanol as modifier with SC-CO2 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 yilded 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 coplete extraction of oleoresin sub-critical propane is more cost-effective than SC-CO2.
3. Extraction of thyme and cardamom seeds by SC-CO2 or sub-critical propane can substantially alter the content and copmposition 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-CO2 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 containlow level of the fat-soluble antioxidant (tocopherols) and low 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 another 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 CO2 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.

**Applicability of new results:**

1. In the field of chemical industries: The Extracts rich in esential oils and natural antioxidants can be used in preparation of several cosmetic products and parfumes. Recently some cosmetic products supplemented with cardamom extracts are favourable for consumers all over the world.
2. In the field of medicine: Since cardamom and thyme products have theraputic effect, their extracts get special interest in the pharmaceutical industries to produce different preparations rich in the effective materials.
3. In the field of food industries and nutrition: Application of cardamom and thyme oleoresins as food additives rich in essential oils and antioxidants is much easier and practical than that of raw material. The extract of both herb can be applied as ingredients in bakeries, dairy products, desserts and salads.

**List of Publication around the topic**

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**Hamdan S**, Daood H.G, Illés V. **(2003**).Compositional changes on chlorophylls and carotenoids as a function of supercritical carbon dioxide and subcritical propane extraction of oregano leaves. Proceedings of the 6th international symposium on Supercritical Fluids TOME 1.2003, 29-30 Aprile 2003, Versailles (France), pp. 353-358

***5 point***

H.G Daood, P Biacs, **S Hamdan**. (**2004**). High-performance liquid chromatography of carotenoids from tomato products on conventional and base-deactivated AB reversed phase column. *In:* International Congress on Pigments in Food, Proceeding. pp. 315-317. ***5 points***

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***1 points***

**Hamdan Samer**, Daood G.Hussein, Toth-Markus Marianna, Illés Vendel. (**2008**).Extraction of cardamom oil by supercritical carbon dioxide and sub-critical propane. Journal of Supercritical Fluids., 44 25-30 ***10 points***

**Hamdan S**, Daood G. Hussein.( **2008**) Changes in the chlorophyll and carotenoid content and composition of ground thyme leaves as a function of supercritical carbon dioxide and sub-critical propane extraction. Acta alimentaria (accepted for publication) ***10 points***

Daood H. G, Korbasz M**, Samer H**, Beczner J. (**2008**). Simultaneous LC determination of Ergosterol, Tocopherols and Carotenoids in foods. Chromatographia (accepted on april 08, 2008). ***10 points***