SUPERCRITICAL FLUIDS – THE CURRENT STATE AND OUTLOOK

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Preface

The CO_2 supercritical extraction process has been in its full bloom for a couple of decades now. Despite the existing limitations, this technology has found its use in different industrial fields. The plant extracts received by the method of supercritical extraction, depending on the type of raw material, are very rich in essential oils, fatty acids, vitamins and polyphenols. The process parameters, such as the temperature and pressure are selected depending on the extract composition constituting the raw material and they are different for the particular raw material.

The supercritical extraction techniques allow for the extract separation during their manufacture phase if multistage separation is used.

Plant extracts can be used in a very wide spectrum and different fields. Plant extract research conducted in many research centers, demonstrated that these extracts exhibit antioxidant, anticancer, antibacterial, antifungal, but also growth stimulating and plant protecting properties as well.

The monograph titled „Supercritical fluid applications" includes the following topics:

Basic research in the field of supercritical extraction

Production, purification and separation of extracts

Methods for analysis of extract composition

Application of extracts in cosmetics, food industry and pharmaceutical industry etc.,

Economic aspects.

The monograph is interdisciplinary. We truly hope that the matters discussed in the monograph will find their appreciation among researchers working in the field of supercritical extraction, manufacturers and those implementing plant extracts on an industrial scale.

Edward Rój
Puławy, October 2016

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RESEARCH ON THE APPLICATION OF SUPERCRITICAL FLUIDS: EXTRACTION, FRACTIONATION, FOAMS, NANOPARTICLES

Summary

The main results of the research in the Laboratory of Supercritical Fluids of the Institute of Chemical Process Fundamentals in Prague involve mathematical models for the kinetics of supercritical fluid extraction from plants, distinguished according to the plants and extracted substances, preparations based on CO_2 extracts for the application as botanical insecticides, and enzymatic reactions of oil in supercritical CO_2 medium. The contemporary research is focused on the on-line fractionation of CO_2 extracts, pressurized liquid extraction, CO_2 assisted foaming of polymers, and recrystallization of the films of metal oxides in water-modified supercritical CO_2.

1. INTRODUCTION

The aim of this contribution was to summarize the research on several applications of supercritical carbon dioxide carried out in the Laboratory of Supercritical Fluids since its establishment more than twenty five years ago. The Laboratory is a part of the Institute of Chemical Process Fundamentals (ICPF), one of the six institutes constituting the section of chemical sciences of the Czech Academy of Sciences. The Institute functions as a centre for advanced research in chemical, biochemical, catalytic and environmental engineering and it acts as a graduate school for PhD. studies in the fields of chemical engineering, physical chemistry, industrial chemistry, and biotechnology.

The research of the Laboratory was initially focused on the mechanisms of supercritical fluid extraction of various substances from plants. In the late eighties, the first large scale facilities were already operating in CO_2 extraction

of caffeine from green coffee beans for the production of decaffeinated coffee and extracts from hops cones to flavour beer. In the case of hops, the capacity of the units for CO_2 extraction was utilized in the months before the next harvest for the extraction of flavors and aromas from other plants and herbs. The first supercritical CO_2 extraction facility operated on a toll basis to meet customers demand for extracts from different plants and herbs was Flavex (Flavex Naturextrakte GmbH), founded in 1986 by people who came from university laboratories where they were involved in the research on the process.

Both design of equipment for new applications of CO_2 extraction and adjustment of extraction conditions in with respect to the varying composition of plant material or to new customers' requirements have been carried out using the experience of operators and the results of experiments conducted in a small scale extraction equipment. We were facing the challenge of developing mathematical models for CO_2 extraction which would effectively supplement these tools.

2. SUPERCRITICAL FLUID EXTRACTION

2.1. Broken and Intact Cell (BIC) models

The extraction conditions (pressure, temperature, feed of plant material and CO_2 flow rate) as well as the pre-treatment (degree of milling of dry material) were systematically varied in the experiments in the laboratory equipment without CO_2 recycle, and mass balance equations for the extraction combined with different equations for mass transfer rate were tested on the data. Simultaneously, the extending literature on modelling the supercritical fluid extraction was monitored. Our experimental results on the extraction of oil from grape seed called for dividing the extraction in two phases, the initial phase controlled by oil solubility in CO_2 followed by a much slower extraction in the second phase. The dependence of the ratio of both extraction periods on the degree of milling indicated that difference in extraction rates is caused by different accessibility of oil in the first and second extraction phases. Starting from the Lack's model [1], which was modified from a model for drying, the BIC model with analytical solution for the extraction yield and concentration profiles in the extractor was derived [2]. The model distinguishes between the extraction of well accessible oil from the cells (cavities) close to particle surface which were opened by milling and the slow extraction controlled by diffusion from intact cells.

Soon it appeared that the BIC model derived for the extraction of oil from seed does not suit other extracted substances and other plant materials because it does not include desorption of solute from plant matrix. As supercritical CO_2 is a week solvent, adsorption equilibrium is usually established between the matrix and the solvent, and only when the contents of extracted substance in the plant is higher than the adsorption capacity, a part of the substance remains free. Perrut

et al. [3] introduced for such case an equation of combined equilibrium, and Reverchon et al. [4] modified the BIC model in such a way that free oil was extracted from open cells and the extraction from intact cells was controlled by adsorption equilibrium. The reason for this linking was minimization of the number of adjustable model parameters; however, it appeared later as a too strong limitation. A more general BIC model was therefore presented and analysed [5] and its simplified versions for different types of supercritical fluid extraction were derived [6].

Three aspects should be defined in a phenomenological model for supercritical fluid extraction: (i) solubility of extracted substance in supercritical CO_2 (equilibrium), which is controlled by extraction pressure and temperature, eventually by CO_2 modification, (ii) mass transfer resistances (dependent on the particle size, and in the case of external mass transfer resistance also on the flow velocity and extraction pressure and temperature), and (iii) flow pattern, which is in ideal case close to plug flow and, in the worst case, channelling causes a rapid decrease in the extraction efficency.

Presently, a BIC model combined with equilibrium function according to Perrut et al. [3] is used in the Laboratory of Supercritical Fluids. The model is sufficiently flexible to simulate different situations which occur in CO_2 extraction and can be easily simplified – e.g. for the extraction of cuticular waxes on the plant surface (all cells in the model are open) or for the extraction of oils from a matrix with low adsorption capacity (the BIC model published in 1994) or for the extraction of substances less abundant in the plant and are completely adsorbed on the plant matrix. Equations of this model are summarized in the Appendix. Together with R.P. Stateva from the Institute of Chemical Engineering of the Bulgarian Academy of Science in Sofia, who specializes in thermodynamic modelling of high-pressure equilibria, we have started modelling of multicomponent extraction of natural products with supercritical CO_2 [7].

2.2. Further research on CO_2 extraction

From the investigation on the mechanisms of supercritical fluid extraction from plants and herbs we proceeded, together with the Crop Research Institute, to the optimization of extraction conditions for botanical insecticides. After extensive screening of the plants grown in mild climates, summer savoury (*Satureja hortensis*) and common rue (*Ruta graveolens*) were found to be the most perspective sources of efficient substances [8]. Further, subtropical plants from South Africa, rich in essential oils, were tested as sources of insecticides [9,10]. The CO2 extracts consist of non-volatile compounds like cuticular waxes and many other substances, and volatile oil, which is almost identical with essential oil – product of steam distillation or hydrodistillation of plants. It was found that the acute toxicity is exhibited in pests by essential oil but higher chronic toxicity, antifeedant activity, and growth inhibition was achieved by

CO_2 extracts from plants, as mixtures of volatile oil and efficient non-volatile compounds.

Presently, the fractionation of extracts integrated with the extraction in one process is studied, and particularly the fractionation of extract solution in supercritical CO_2 by adsorption under pressure [10]. Another studied topic is the pressurized solvent extraction, where the solvent is by pressure maintained in liquid state at temperatures higher than its boiling temperature at normal pressure. The pressurized solvent extraction is much faster and more efficient than the extraction with supercritical CO_2 but it is at the expense of low selectivity. Thus, a combination of both extraction methods opens new possibilities in obtaining isolates from plants and herbs.

We have started cooperation with Czech company Trumf International, s.r.o., which uses a high capacity extractor for supercritical CO_2 extraction of spices.

3. ENZYMATIC REACTIONS IN CO_2

It was a short way from the supercritical fluid extraction to reaction of substrates which were dissolved in supercritical CO_2 and the solution flowed through a bed immobilized enzyme. In cooperation with the Institute of Organic Chemistry and Biochemistry, another institute of the section of chemical sciences of the Czech Academy of Sciences, we focused on the hydrolysis of black currant seed by different lipases, out of which the best performance in supercritical CO_2 was that of commercial Lipozyme. The aim of the project was to carry out a partial oil hydrolysis using the enzyme specificity, releasing from the glycerol skeleton of oil molecules preferentially one of two polyunsaturated fatty acids, α- and γ-linolenic acid, which are equally abundant in the oil. Two products with different ratio of both fatty acids would be obtained, which could be, thanks to the absolutely clean process applied, directly administered to patients according to their needs, either to supplement ω-3 fatty acids or ω-6 fatty acids.

The regiospecific Lipozyme was found to release preferentially α-linolenic acid from the primary positions in the molecules of black currant seed oil, while γ-linolenic acid remained bound in the secondary position. The enzymatic reaction in supercritical CO_2 was fast, the necessary 30% conversion was achieved with residence time about 4 min [11]. It took a longer time to solve the problem with enzyme inactivation after several cycles of pressurizing /depressurizing, however, also this bottleneck seems to be overcome [12]. The grant was, however, over before we tackled the last task – a clean separation of the reaction mixture of unreacted triglycerides, partial glycerides, and free fatty acids.

Even today we connsider the enzymatic reactions in supercritical CO_2 catalyzed by Lipozyme and some other immobilised lipases as a very promising process. Its perspective of industrial application is sometimes doubted with regards to the danger of rapid inactivation of the enzyme but, according to our

experience, the inactivation can be suppressed when certain conditions of reaction and depressurisation are maintained.

4. FOAMING OF POLYMERS WITH SUPERCRITICAL CO_2

Polymer foams are especially used as thermal and sound insulators, food packaging and cushioning material. Beside the conventional foam production, the foam production using CO_2 appears to be perspective. CO_2 under pressure and at elevated temperature dissolves in the polymer and during a rapid depressurization forms bubbles and, thus, induces foaming. For good thermal insulation properties, a high foam porosity (more than 90%) and small bubble size (around 1 μm and less) are required. The foaming with CO_2 has been studied by a number of researchers and its mathematical description is getting more and more precise. The published mathematical models include the nucleation rate according to the nucleation theory and the rate of bubble growth. They enable to calculate the bubble density, bubble size and, in case of nucleation occurring also during the bubble growth, distribution of bubble sizes in the foam [13]. We have attempted to describe the foam at the moment when the polymer vitrifies because its temperature falls to T_g, the glass transition temperature. The mixture of polymer and CO_2 is cooled down by the expanding gas. The foam porosity can be estimated on the basis of heat balance when the pressure- and temperature dependence of CO_2 solubility in the polymer and the pressure dependence of T_g are known.

Experiments with the foaming of polystyrene films of 0.11–1.2 mm thickness at different saturation pressures and temperatures were conducted in cooperation with the University of Chemistry and Technology Prague. In contrast to the foaming of larger particles of polymer, the cooling of the thin film surface by the expanding CO_2 outside the film could not be neglected in the estimations of foam porosity, and a porosity profile with a minimum at the surface was measured and predicted. As a result of the outer cooling of the film, the foam porosity was relatively low.

The next experimental runs were carried out using light hydrocarbons as polystyrene modifiers; a substantial increase of the foam porosity was achieved.

5. CRYSTALLIZATION IN MODIFIED CO_2

Titanium dioxide, particularly in the anatase form, is a photocatalyst under UV light. It can be used in a form of thin films in gas sensors, as coatings for self-cleaning surfaces or and/or surfaces with antimicrobial activity, electrode material, a photocatalyst promising in waste water and air treatment technologies, and in other application areas. Commonly used method for the preparation of pure and crystalline TiO_2 thin films is calcination; however, this

standard thermal processing was overcome by supercritical carbon dioxide drying combined with thermal processing. The motivation of the research carried out in the Laboratory of Supercritical Fluids in cooperation with the Institute of Environmental Technology, VŠB-Technical University of Ostrava, is to design an improved supercritical fluid process which can lead to purification and direct crystallization of TiO_2 thin films without any subsequent thermal treatment. Processing by pure and water-modified (30 wt.%) supercritical carbon dioxide and by subcritical water were utilized for the direct preparation of highly pure TiO_2. One-step processing was compared with the two- or three-step processing combining pure and modified CO_2, and the effect of temperature (40–150°C) and the amount of CO_2 passed across the thin films on their (micro)structure and purity were examined at pressure of 30 MPa [14]. The optimum processing conditions were determined and the results of evaluation of photocatalytic activity of these films are presently expected.

6. CONCLUSIONS

Though the majority of the results of the research in the Laboratory of Supercritical Fluids in the Institute of Chemical Process Fundamentals in Prague is still from the field of supercritical fluid extraction od plants and herbs, new directions of research were opened up in the last years. The heading of the boat of our research to new areas is less dependent on the steer adjustment – it is on our intentions – than on the sea streams, it is on the success or failure in the competition for financial support of research projects. Our priorities are the application of supercritical fluid extraction and fundamental research in new areas of supercritical CO_2 utilization.

APPENDIX
BIC model with solute desorption from plant matrix

Phenomenological model for the extraction from particles of plant material in a semi-continuous set-up is based on mass balance equations for the fluid phase and for two regions in the particles, the region of open cells and the region of intact cells. The extraction column is axially divided in n ideal mixers of equal size and a set of $3n$ ordinary differential equations is formed:

$$\frac{dy_j}{dt} = \frac{y_{j-1} - y_j}{t_r} + \frac{y_j^+ - y_j}{t_f}$$

$$r\frac{dx_{Bj}}{dt} = -\gamma\frac{y_j^+ - y_j}{t_f} + \frac{x_{Ij} - x_{Bj}}{t_s}$$

$$(1-r)\frac{dx_{Ij}}{dt} = -\frac{x_{Ij} - x_{Bj}}{t_s} \quad \text{for } j=1,2,\ldots,n \qquad \text{(A1)}$$

where y_j is the extract concentration in the fluid phase in the j-th mixer (kg/kg CO_2), x_{Bj} is the concentration of the extracted substance in the region of broken cells (kg/kg solid phase), x_{Ij} is the concentration of the extracted substance in the region of intact cells (kg/kg solid phase), t is the extraction time counted from the beginning of the dynamic extraction (s), r is the fraction of the region with open cells in the particles, γ is the CO_2-to-solid phase mass ratio in the extraction bed (kg/kg), t_r is the residence time (s), and t_f, t_s are the characteristic times of mass transfer resistances in the fluid phase and in the region with intact cells, respectively (s):

$$t_f = \frac{\varepsilon}{k_f a_0}, \; t_s = \frac{1-\varepsilon}{k_s a_0} \tag{A2}$$

The mass transfer resistance in open cells is neglected. The initial and boundary conditions are

$$y_j = y_0, \quad x_{Bj} = x_{B0}, \quad x_{Ij} = x_u, \quad \text{for } i = 1,2,\ldots,n$$
$$y_{j-1} = 0, \quad \text{for } j = 1 \tag{A3}$$

The content of solute in the material inserted in the extractor is x_u (kg/kg solid phase). While no mass transfer is assumed from the intact cells before the dynamic extraction begins, a certain amount of solute, which depends on the time of static extraction, can be transferred from open cells to the fluid phase. The initial mass balance is therefore

$$r\left(x_u - x_0\right) = \gamma y_0 \tag{A4}$$

The phase equilibrium is described using the function according to Perrut et al. [3]:

$$y_j^+ = \begin{cases} y_s & \textit{for} \;\; x_j \geq x_t \\ K x_{Bj} & \textit{for} \;\; x_j < x_t \end{cases} \tag{A5}$$

Eq. (A5) is written for a matrix which adsorption capacity is equal to x_t (kg/kg solid). When the plant contains higher amount of solute, a part of it is free and is dissolved in CO_2 with the solubility y_s (kg/kg CO_2).The equilibrium fluid phase concentration of the remaining solute, which is adsorbed on the plant matrix, is defined by the partition coefficient K (kg solid/kg CO_2).

All equilibrium parameters, ys, xt and K, are dependent on extraction temperature and pressure.

The extraction yield, e (kg/kg plant), is calculated from the concentration in the last mixer:

$$\frac{de}{dt} = q'y_n, \quad e(t=0) = 0 \tag{A6}$$

where q' is the specific mass flow rate (kg CO_2/kg plant . s), $\gamma = q't_r$.

The model can be simplified for various specific situations: easily accessible solute when $r = 1$ or no broken cells ($r = 0$), all solute adsorbed on plant matrix when $x_u < x_k$ or negligible adsorption when $x_k \rightarrow 0$. The degree of axial mixing is controlled by the number of mixers. A short extractor can be modelled with $n = 1$, as a mixer. On the other hand, the plug flow pattern is approached with a large number of mixers.

Should the discontinuity in the equilibrium function, eq. (A4), complicate the integration of differential equations, it can be substituted by a continuous function proposed by del Valle et al. [15]:

$$y_j^+ = Kx_j + 1/(1+(x_t/x_j)^b)(y_s - Kx_j) \tag{A7}$$

with exponent b controlling the steepness of the transition from dissolution of free solute to solute desorption from plant matrix.

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ANALYSIS OF SUPERCRITICAL CARBON(IV)OXIDE EXTRACTS FROM SELECTED PLANTS

Summary

The extraction of natural products with supercritical carbon dioxide ($ScCO_2$) has found numerous large-scale applications in the food and perfume industries including the decaffeination of coffee beans, the extraction of bitter principles from hops, removal of essential and pungent principles from spices, extraction of natural food colorants and essential oils for perfumery, due to some inherent advantages of supercritical fluid extraction (SFE) over conventional solvent extraction. Taking into consideration that a vast number of research on chemical constituents in the extracts obtained by conventional extraction methods have been conducted, we investigated the extracts obtained applying SFE with carbon(IV)oxide as a solvent, using GC techniques for analytical scales research. Although the lipophilic nature of $ScCO_2$ represents one of major limiting factors, in a case of extraction of lipophilic secondary metabolites from the medicinal plants, using the $ScCO_2$ as a solvent might be the choice of matter. In this paper the chemical analysis of lipophylic fraction present in $ScCO_2$ extracts from selected plants (*Melissa officinalis* L., Lamiaceae, *Nigella sativa* L., Ranunculaceae, *Matricaria chamomilla* L., Asteraceae, *Urtica dioica* L., Urticaceae, *Humulus lupulus* L., Cannabaceae, *Cannabis sativa* L., Cannabaceae), was performed applying GC-MS and GC-FID techniques, stressing the importance of identified compounds for pharmaceutical application. The goal of the performed analysis was to give evidence of the chosen classes of secondary metabolites (terpenes, fatty acids and terpenophenolics) presence in investigated $ScCO_2$ extracts.

1. INTRODUCTION

With several hundreds of published scientific papers, the application of supercritical fluids (SCFs) for obtaining the compounds from natural sources represents one of the most widely studied extraction techniques. Supercritical fluids extraction (SFE) has immediate advantages over traditional extraction techniques: it is a flexible process due to the possibility of continuous modulation of the solvent power/selectivity of the SCF, allows the elimination of polluting organic solvents and avoids the expensive post-processing of the extracts for solvent elimination. 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, odourless, tasteless, environment-friendly, and GRAS (generally regarded as safe) solvent. In SCF processing with CO_2, there is no solvent residue in the extract, since it is a gas in the ambient condition. In addition, its near-ambient critical temperature (31.1°C) makes it ideally suitable for thermolabile 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 [1]. Taking into account the structural complexity and variability (with the season, kind, crop, etc.) of the materials to be treated and the large variety of compounds that can be extracted (different molecular weight, polarity, link with the structure, etc.), the ScF extracts are far from to be considered exhaustively studied, though some industrial applications have been already developed. Moreover, an increasing interest has been registered in the extraction of high added value substances, such as antioxidants, pharmaceuticals and colouring substances.

Herbal medicine has been proven to be an effective therapy offering a variety of benefits, such as moderate reduction in manifestation of different health impairments due to the oxidative stress. According to modern theory of free radical biology and medicine, reactive oxygen species are involved in several disorders. The harmful action of the free radicals can be, however, blocked by antioxidant substances which scavenge the free radicals and detoxifying the organism. Current research into free radicals has confirmed that medicinal plants and foods rich in antioxidants play an essential role in the prevention of cancers, cardiovascular and neurodegenerative diseases. Of raised interest are essential fatty acids and terpenes as non-polar compounds and specific compounds from mixed biogenesis involving the terpene pathways, like phloroglucinol and orcinol derivatives (bitter acids and cannabinoids). The chemical nature of the mentioned groups of compounds allows application of $ScCO_2$ as extracant in obtaining the extracts with standardized and high quantity of mentioned physiologically active compounds. However, sometimes non-polar compounds might represent the impurities undesirable in final extract formulation, and applying $ScCO_2$ extraction for separation the active part of the extracts in such cases proved to be useful, as well.

Terpenoids represent the class of secondary metabolites with broad range of biological activities, and therefore the establishing the procedure of selective extraction might be of the great importance. Namely, these secondary metabolites can act as defensive substances in plants (allomones) and animals, they can be used by plants to deter herbivores or to inform conspecifics, or attract natural enemies of herbivores (synomones). Their effects as internal messengers might be additive or even synergistic with other mevalonate metabolites or they are inhibitors of parts of mevalonate pathway. So, the mode of their activity should be viewed with the respect to the role of other mevalonate metabolites. The co-extraction of non-volatile terpenoids, concurrently with plant sterols, fatty acids or other lipophylic compounds might be interesting in explanation of overall activity ascribed to tested plants. Hence, the principles of their physiological role in organism where they are produced might be considered as base to combat complex pathological disorders in mammals (and man), such as developing cancers. Combination and multi-stage therapies (with terpenoids already known to have a function in e.g. plant-insect relationships) show a great deal of analogy with the strategies utilized by plants to defend themselves from attack by micro-organisms and herbivores. Plant hormones are often derivatives of terpenoids, such as cytokinins, gibberellins and abscisic acid. The steroid hormones of mammals are terpenoids with an advanced but not very complex structure. Although high vegetable, fruit and cereal intake in general tend to offer a better protection against cancers of the alimentary and respiratory tracts than to hormone-sensitive cancers the increased intake of specific medicinal plant as preventive step could become an attractive approach to prevent the development of malignancies in individuals with increased congenital risks. This may hold for antioxidants such as ascorbic acid, selenium ions and isoprenoids like vitamin E, vitamin A and β-carotene as well as for more specific metabolites. Examples are lycopene, specific carotenoids and retinol in relation to prostate cancer [2]. Hence, having in mind inexhaustible diversity of secondary and primary metabolites present in plants, discovering new molecules with significant biologically activities might be the goal of today's natural compounds investigation.

Sterols are ubiquitous and essential membrane components found in all eukaryotes. They regulate membrane fluidity and permeability and interact with lipids and proteins within the membranes. They are also precursors of a vast array of bioactive compounds involved in important cellular and developmental processes, and plant sterols are particularly linked to brassinosteroid synthesis [3].

Phloroglucinol derivatives are secondary metabolites that occur naturally in certain plant species. Formylated phloroglucinol compounds (euglobals, macrocarpals and sideroxylonals) can be found in *Eucalyptus* species [4], hyperforin and adhyperforin are two phloroglucinols found in St John's wort (*Hypericum perforatum* L., Hypericaceae). The activities attributed to St John's wort may be due to the plant's use of interacting phytochemicals to accomplish

many complementary tasks, among them being the hyperforins, a family of prenylated acylphloroglucinols known for their multitarget activities [5–7]. Bitter-tasting phloroglucinol derivatives known as bitter acids, with isoprenoid side-chains, were found in the resin of mature hops (*Humulus lupulus* L., Cannabaceae). It is known that antioxidative activity of hop bitter acids and their analogues may contribute to a cancer preventive effect since they can quench free radicals which cause oxidation of the DNA in the body and thus cause genetic defects. Humulon has been known for its preventive effect against osteoporosis and for capability to hinder the growth of certain leukemia cells especially with the combination of vitamine D. The results of one recent study confirmed that beer components are protective against the genotoxic effects of heterocyclic amines on target organs associated with tumorigenesis „in vivo". Also, it is known that isomerized bitter acids (isohumulones) might prevent the developments of noninsulin dependent diabetes and hyperlipidemia, improving insulin sensitivity in patients with type II diabetes [8, 9].

Cannabinoids, a group of terpenphenolics, are accumulated in considerable amounts in glandular trichomes of *Cannabis sativa* L., Cannabaceae. Numerous representatives of this group have been characterized. Because of their psychotomimetic effects, *Cannabis* sp. preparations such as marijuana and hashish, have been used for centuries and are still among the most widely used illicit drugs. Since the discovery of specific receptors for tetrahydrocannabinol in mammalian brain and peripheral tissues, and the isolation of endogenous ligands for these receptors, cannabinoids have attracted renewed interest for medicinal applications including the relief of pain, nausea caused by cancer chemotherapy or acute glaucoma, and the control of spasticity and tremor in patients suffering from multiple sclerosis, as well as for therapy of arthritis [10, 11].

The goal of this work is to present the chemical fingerprint of selected medicinal plants extracts obtained applying $ScCO_2$, with the special attention to non-volatile, non-polar constituents, readily extracted from the plant material. The presence of terpenoids, especially diterpenes, triterpenes and phytosterols, fatty acids and its derivatives in investigated $ScCO_2$ extracts of selected plants (*Melissa officinalis* L., Lamiaceae (lemon balm), *Matricaria chamomilla* L., Asteraceae (German chamomile), *Urtica dioica* L., Urticaceae (stinging nettle), *Nigella sativa* L., Ranunculaceae (black caraway, black cumin), and phloroglucinol and orcinol derivatives (as the main constituents of *Humulus lupulus* L. (hops), and *Cannabis sativa* L., Cannabaceae (hemp)) were discussed taking into consideration their biological activities. In addition, the significance of applying the Sc CO_2 extraction as mean of selective isolation of fractions rich in mentioned groups of compounds has been stressed. For the aim to compare the chemical composition of the crude $ScCO_2$ extracts and the extract obtained by further purification applying the Sc extraction process, hemp extracts were

analyzed and mutually compared. Chemical analysis of obtained $ScCO_2$ extracts was performed using GC-MS and GC-FID spectrometry methods, being chosen because of the simplicity of the procedure which enables identification of each compound based on its MS spectrum.

2. MAIN PART

2A. Materials and methods

The investigated $ScCO_2$ extracts were obtained from Supercritical Extraction Deaprtment, Instytut Nowych Syntez Chemicznych, Puławy, Poland (*M. chamomilla*-German chamomile, *U. dioica*-stinging nettle, *N. sativa*-black carway, black cumin, *H. lupulus*-hops and *C. sativa*-hemp 1, crude extract), BAFA neu GmbH, Germany (*C. sativa* – hemp 2, purified $ScCO_2$ extract) and Faculty of Technology and Metallurgy, University of Belgrade, Serbia (*M. officinalis*-lemon balm). The extracts were dissolved in mixture of chloroform and methanol, enabling the thorough dissolution of the extracts in the chosen media. The final concentrations of the investigated extracts were 30.2, 31.9, 35.3, 36.7 and 37.8, 37.3, and 45.0 mg/mL, for black caraway, German chamomile, peppers, hops, hemp-extract 1 (crude $ScCO_2$ extract) and hemp-extract 2 (purified $ScCO_2$ extract), and lemon balm, retrospectively.

Gas chromatography analysis of the investigated $ScCO_2$ extracts was carried out on a HP-5890 Series II GC apparatus [Hewlett-Packard, Waldbronn (Germany)], equipped with split–splitless injector and automatic liquid sampler, attached to HP-5 column (25 m×0.32 mm, 0.52 μm film thickness) and fitted to flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:5, injector temperature was 250^{o}C, detector temperature 300^{o}C, while column temperature was linearly programmed from 40 to 300^{o}C (at rate of 4^{o}C /min), and then kept isothermally at 300^{o}C for 30 min. Solutions of samples in chloroform/methanol mixture were consecutively injected in amount of 1 μl. Area percent reports, obtained as result of standard processing of chromatograms, were used as base for the quantification analysis.

The same analytical conditions as those mentioned for GC-FID were employed for GC-MS analysis, along with column HP-5MS (30 m×0.25 mm, 0.25 μm film thickness), using HP G 1800C Series II GCD system [Hewlett-Packard, Palo Alto, CA (USA)]. Helium was used as carrier gas. Transfer line was heated at 260^{o}C. Mass spectra were acquired in EI mode (70 eV); in m/z range 40–450. The amount of 0.2 μl of sample solution in chloroform/methanol mixture was injected. The components of the extracts were identified by comparison of their mass spectra to those from Wiley 275 and NIST/NBS libraries, using different search engines. Identification of the compounds were

achieved by comparing their retention indices and mass spectra with those found in the literature [12] and supplemented by the Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver. 2.1), GC-MS Libraries [13]. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System Software (AMDIS ver. 2.1), GC-MS Libraries [13], compared to those from available literature (Adams 2007) [12] and used as additional tool to approve MS findings.

The relative proportions of the constituents were expressed as percentages obtained by peak area normalization, all relative response factors being taking as one.

2B. Results and discussion

The use of mass spectrometric detection with its excellent capability for the determination of compound chemical formula makes GC-MS an irreplaceable tool when analysis requires compound identification. Although significant progress has been made in using LC/MS (and LC/MS/MS) for compound identification, these techniques still remain more adequate for quantitation and not for qualitative analysis. The GC-MS analysis has considerable advantages compared to other analytical techniques. Besides the simplicity of the procedure, the technique can be used for definite identification of each compound based on its MS spectrum. The disadvantage of the technique is caused by the need for volatility and certain thermal stability for the compounds to be analysed. However, several procedures are used for extending the capability of gas chromatography for the analysis of non-volatile and larger molecules. Among these procedures are specific techniques for sample preparation (derivatization of the analytes). Other procedures include certain GC instrument settings such as the use of hydrogen as carrier gas, selection of appropriate chromatographic column, selection of the type of injection port, and a GC oven gradient with high final temperatures, etc. The typical split/splitless injection port, with relatively high temperatures (e.g. around 300^{o}C) is frequently adequate for the analysis of larger molecules.

The set of specific conditions enabled the analysis of non-volatile compounds present in the investigated $ScCO_2$ extracts. To avoid the cumbersome tables, only identification of the target compounds were presented, and graphs were given within which the quantitative and qualitative comparison of the targets compounds' groups were obvious (Figure 1, Table 1). The investigated plants' extracts were chosen due to their chemical composition and their significant antioxidant potential documented in the literature data and corresponding application in pharmaceutical, cosmetic and food industries [7, 14–19].

Table 1. The results of GC-MS analysis of investigated ScCO2 extracts *M. officinalis, M. chamomilla, U. dioica, N. sativa, H. lupulus,* and *C. sativa*, extract 1 and extract 2 (only selected components with interest for the presented work were given)

***Melissa officinalis* L., Lamiaceae**		***Matricaria chamomilla* L., Asteraceae**		***Urtica dioica* L., Urticaceae**		***Nigella sativa L.*, Ranunculaceae**	
Identified non-volatile compounds	**(%)**	**Identified non-volatile compounds**	**(%)**	**Identified non-volatile compounds**	**(%)**	**Identified non-volatile compounds**	**(%)**
hexadecanoic acid	14.2	methyl hexadecanoate	2.7	methyl hexadecanoate	1.2	methyl hexadecanoate	0.3
9,12-octadecadienoic acid *(Z,Z)* methyl ester	4.6	9,12-octadecadieonoic acid (Z,Z), methyl ester	12.5	hexadecanoic acid	4.2	pimara-8,15-diene	0.5
octadecanol	2.6	9,12-octadecadieonoic acid (Z,Z)	1.3	methyl 9-octadecenoic acid	0.7	hexadecanoic acid	22
9,12-octadecadienoic acid (Z,Z)	13.6	9,12-octadecadieonoic acid (Z,Z), ethyl ester	0.4	phytol	22.5	phytol	0.1
abietinol	3.0	9,12-octadecadieonoic acid (Z,Z), isopropyl ester	0.2	methyl octadecanoate	1.3	3,7,11,15-tetramethyl-2-hexadecen-1-ol	0.3
trans-ferruginol	2.6	tricosane	0.7	9,12-octadecadienoic acid	8.5	9,12-octadecadienoic acid (Z,Z), methyl ester	0.1
labd-7,13-dien-15-yl acetate	0.9	tetracosane	0.2	ethyl ester 9,12-octadecadienoic acid	0.3	9- octadecenoic acid (Z)	0.2
tetracosane	4.1	erucic acid	0.2	9-octadecenoic acid	0.7	octadecanoic acid, methyl ester	0.2
pentacosane	1.9	hexacosane	10.7	phytol acetate	3.9	9,12-octadecadienoic acid (Z,Z)	67.6
heptacosane	5.2	tetracosanol-1	0.1	tricosane	0.9	9,12-octadecadienoic acid (Z,Z), ethyl ester	0.1
nonacosane	2.2	1-monolinolein	0.2	tetradecyl cyclooctane	2.9		
untriacontane	4.3	heptacosane	0.6	pentacosane	0.2		
dotriacontane	2.2	octacosane	3.5	heptacosane	1.1		
(3b, 4S)-stigma-5-en-3ol	6.3	nonacosane	1.5	squalene	0.3		
tetratricanonatne	4.7	hentriacontane	0.2	octacosane	1.5		
		methyl hentriacontane	0.1	hentriacontane	0.2		
		β-sitosterol	0.3	campesterol	0.4		
		3β-cholest-5-en-3-ol, 4,4-dimethyl	0.3	β-sitosterol	2.5		
		cholest-4,6,8(14)-triene	0.1	β-amyrin	1.8		
		cycloartenyl acetate	0.3	α-amyrin	1.8		
				3-O-acetyl-δ-24-cycloartenol	0.1		

Table 1(cont). The results of GC-MS analysis of investigated ScCO2 extracts *M. officinalis, M. chamomilla, U. dioica, N. sativa, H. lupulus,* and *C. sativa*, extract 1 and extract 2 (only selected components with interest for the presented work were given)

Humulus lupulus L., Cannabaceae		Canabis sativa L., Cannabaceae, extract 1		Cannabis sativa L., Cannabaceae, extract 2	
Identified non-volatile compounds	**(%)**	**Identified non-volatile compounds**	**(%)**	**Identified non-volatile compounds**	**(%)**
dehydrocohumulinic acid	3	methyl hexadecanoate	0.2	dehydrocohumulinic acid	0.2
dehydroisohumulinic acid	1.7	dehydrocohumulinic acid	0.2	cembrene	0.2
dehydrohumulinic acid	7.5	hexadecanoic acid	0.3	dehydroisohumulinic acid	0.1
ethyl linoleate	0.8	dehydroisohumulinic acid	0.9	dehydrohumulinic acid	0.3
methyl squalene	0.7	dehydrohumulinic acid	11	3,7,11,15-tetramethyl-2-hexadecen-1-ol	0.1
trans-isohumulone	2	3,7,11,15-tetramethyl-2-hexadecen-1-ol	0.5	methyl ester 9,12-octadecadieonoic acid	0.5
cis-isohumulone	0.6	methyl ester 9,12-octadecadieonoic acid	1.4	9,12,15-Octadecatrienoic acid, methyl ester	0.3
humulone	0.5	9,12,15-octadecatrienoic acid, methyl ester	1.9	phytol	0.4
6α-acetoxy-4-propyl-3,4-seco-5β-androstene-3,17α-diol	5.9	phytol	0.8	propylcannabidiol	2.1
lupulone	8.6	9,12-octadecadienoic acid	59.3	cannabichromene	2.6
pregnan-3-one-17-hydroxy-5α,17α	2.1	lupulone	0.4	cannabicitran	0.5
23-methyl-cholesta-5,22(Z)-dien-3β-ol (398)	0.7	cannabidiol	6.6	cannabidiol	67
tetrahydrosqualene	0.3	cannabipinol	0.7	α-cannabielsoin	0.7
Brassicasterol	0.2	cannabigerol	0.3	cannabigerol	0.2
23,24-dimethyl-cholesta-5β,22(E)-dien-3-ol	0.6	hexahydocannabi-1,10β-diol	0.2	cannabinol	0.3
		cholesta-3-ol-2-methylene (3β,2α)	0.1	squalene acetate	0.3
		squalene	0.8	Vit. E	0.4
		Vit. E	t	β-sitosterol	t
		β-sitosterol	0.5	β-amyrin	t
		β-stigmasterol	t	α-amyrin	t
		β-amyrin	t	3α-12-oleanen-3-yl acetate	t
		α-amyrin	t	β-sitosterol acetate	t
		3α-12-oleanen-3-yl acetate	t	cycloartenyl acetate	t
		cycloartenyl acetate	t	3-oxo-urs-12-en-24-oic acid, methyl ester	t
		3-oxo-urs-12-en-24-oic acid, methyl ester	t	lupeol acetate	t
		cycloartenol	t	cycloartenol	t

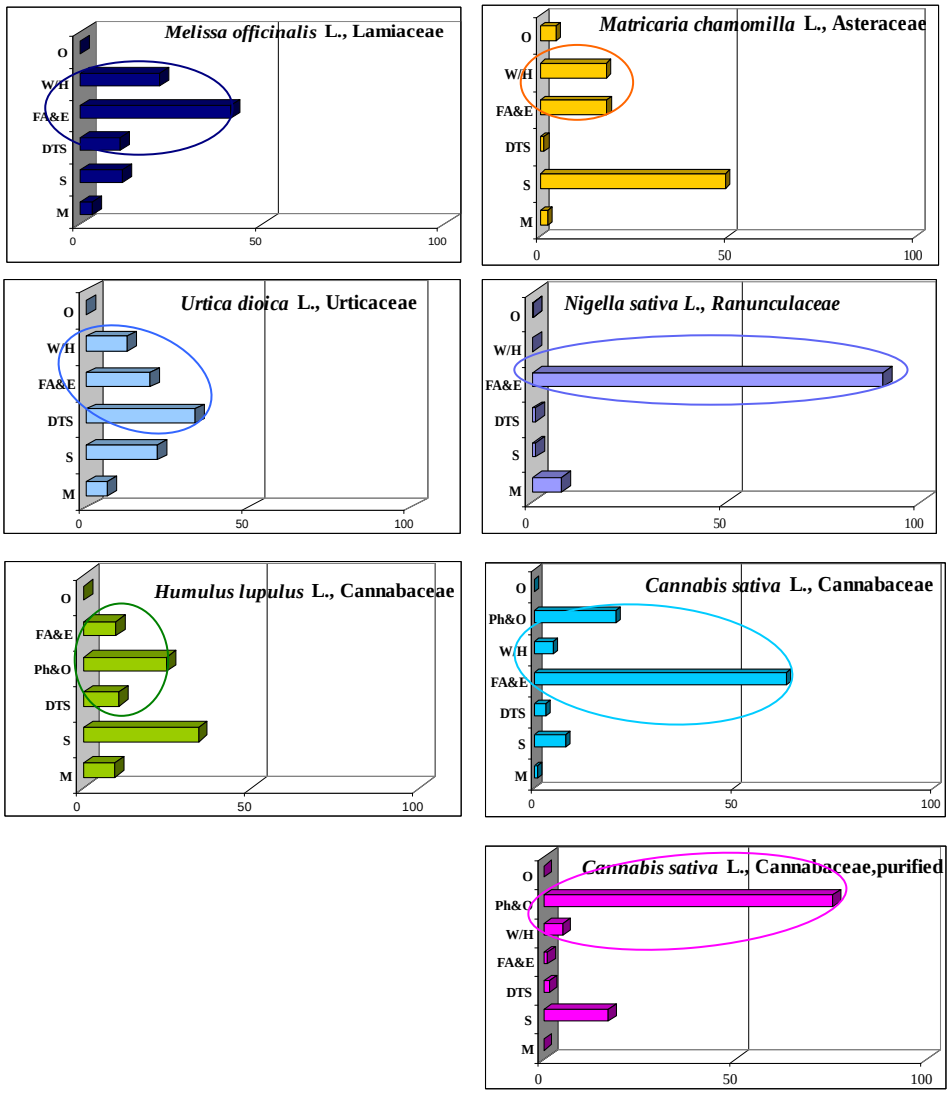


Fig. 1. Comparative representation of particular groups of compounds (monoterpene hydrocarbons and oxygenated monoterpenes (M), sesquiterpene hydrocarbons and oxygenated sesquiterpene (S), nonvolatile terpenoids (diterpenoids, triterpenoids, steroids -DTS), fatty acids&esters&aldehydes &alcohols (FA&E), Waxes/Hydrocarbones (W/H), Phloroglucinol&orcinol derivatives (Ph&O) and others, non belonging to above mentioned groups of secondary metabolites (O) in the investigated extracts obtained by supercritical carbon dioxide extraction - $ScCO_2$

Lemon balm (*M. officinalis*) is an aromatic perennial herb being used for more than 3000 years in the Tibetan medicine as bioactive plant against psychological problems. Nowadays effects of orally admission of lemon balm are under investigation in the case of anxiety, insomnia, dyspepsia, vomiting,

bloating, attention deficit-hyperactivity disorder (ADHD), dysmenorrheal, cramps, headache, toothache, anesthetic, infections, tumors, insect bites, Alzheimer's disease, hysteria, melancholia, colic, palpitations, rheumatism, and high blood pressure. The German Commission E approves lemon balm for nervous disorders, as a soporific and in the cure of gastrointestinal disorders. The traditional European medicine comprises the use of lemon balm as tea for hysteria, migraine, toothache, headache and high blood pressure [7, 20]. In phytotherapy, the lemon balm is known for its carminative, gastric, calmative, antiseptic, antiviral, antimicrobial, and antioxidative properties. Recently, this plant has been studied for its interesting hypolipidemic effect [21–24].

Our results indicated that $ScCO_2$ extraction gave the extract rich in fatty acids and their derivatives, what was in accordance with recently published results regarding the different conditions applied in Sc fluid extraction in order to obtain the fraction of this plant rich in antioxidant substances (Figures 1, 2, 3; Table 1) [14]. Namely, the investigated extract contained hexadecanoic and 9,12-octadecadienoic acids (ω-6 PUFA) as the major compounds (16.9 and 20.2%, respectively), with significant percentage of its esters. Interestingly, diterpenes were present in smaller quantity, but not insignificant, and the GC-MS analysis revealed presence of *trans*-ferruginol, abietinol and labd-7,13-dien-15-yl acetate (2.8, 2.1 and 2.6% respectively) (Table 1, Figure 2). Naturally occurring diterpenes exert several biological activities such as anti-inflammatory, antimicrobial and antispasmodic activities. Labdane, pimarane and abitane diterpenes have been shown to possess activity against human liver carcinoma [25]. Besides, the ferruginol derivatives might be interesting as substances with antimalarial potential [26]. Interestingly, it was suggested that the hexadecanoic acid might function as an anti-inflammatory agent, as it had shown significant inhibitory activity of phospholipase A2 in the enzyme kinetics study, validating the rigorous use of medicated oils rich in n-hexadecanoic acid for the treatment of rheumatic symptoms. Fatty acids can modulate immune responses by acting directly on T cells. The dietary, conjugated linoleic acid exerts anti-inflammatory effect by decreasing production of the inflammatory mediators such as prostaglandin E2, IL-6, IL-1b, TNFa, and nitric oxide [27]. The presented results regarding the application of $ScCO_2$ extraction in a case of this medicinal plant opened the possibility to investigate the conditions for controlled extraction, meaning that certain fractions rich in specific compounds recognised as substances contributing or enriching the traditionally known application, might be obtained. Therefore, this finding might focus further investigation of lemon balm $ScCO_2$ extraction to different secondary metabolites, taking into account that mainly scientific attention up to now has been directed to rosmarinic acid as active and pharmacologically potent compound. The chemical nature of the identified compounds is of scientific concern, according to abovementioned facts about the pharmacological activities ascribed to those classes of secondary metabolites. Comparative study of composition's profile of lemon balm extracts obtained by different methods of

extractions ($ScCO_2$ and conventional techniques) might help to define the SCO_2 extraction conditions for obtaining the extracts with desirable chemical profile. Conducting the research on different ScF extraction parameters, various types of the extracts might be obtained depending on desirable final effect and intended application in food, cosmetic or pharmaceutical industries.

German chamomile (*M. chamomilla*) is a well-known medicinal plant often referred to as the "star among medicinal species." Nowadays it is a highly favored and much used medicinal plant in folk and traditional medicine. Its multitherapeutic, cosmetic, and nutritional values have been established through years of traditional and scientific use and research (7). Chamomile possesses significant antiinflammatory and antiseptic, also antispasmodic and mildly sudorific properties. It is used internally mainly as infuse (1 table-spoonful of the drug in 1 L of cold water, without heating) for disturbance of the stomach associated with pain, for sluggish digestion, for diarrhea and nausea; more rarely and very effectively for inflammation of the urinary tract and for painful menstruation. Externally, the drug in powder form may be applied to wounds slow to heal, for skin eruptions, and infections, such as shingles and boils, also for hemorrhoids and for inflammation of the mouth, throat, and the eyes. Chamomile extraction isolates, such as apigenin and its 7-glycoside derivatives, α-bisabolol and its A and B oxides, chamazulene, matricine and en-indicycloethers, are known to be antiinflammatory and antioxidative. They are used topically for the treatment of both acute and chronic skin diseases, including contact dermatitis, eczema, wound healing and damage caused by irradiation [7, 18]. Chamomile is a rich source of terpenoids and flavonoids and can possess anti-inflammatory, antioxidant and anti-nociceptive effects. In addition, chamomile is a safe medicinal herb, and especially there were several reports on its external use for a variety of diseases on different sites of the human body. German chamomile is listed as "FDA's generally recognized as safe" herb [28].

The traditional methods for extraction of this plant material as steam distillation, organic solvent extraction using maceration or Soxhlet techniques have distinct drawbacks such as high temperature and extended concentration steps which can result in the loss or degradation of volatile components in the extract. Reverchon and Senatore used supercritical CO_2 to obtain chamomile extracts. These authors verified that the SFE produced chamomile extracts contained both essential oil and cuticular wax, due to non-selectivity of CO_2 [29]. To gain stepwise fractionation of the obtained $ScCO_2$, some authors used low temperature extraction conditions and pressure up to 200 bar achieving the fractional separation of essential oil and waxes [30]. Our results revealed that $ScCO_2$ extract of German chamomile contained, except the fraction rich in volatile sesquiterpene and polyynes, fractions of fatty acids and higher hydrogencarbones, in percentage of 17.5 and 17.6, respectively (Table 1, Figures 1, 3). Methyl linoleate was determined to be mostly present, with significant quantity of higher hydrogencarbon hexacosane (12.7 and 10.5%, respectively).

Worth mentioning, in the investigated sample the presence of sterols was detected, as well (β-sitosterol, 3β-cholest-5-en-3-ol, 4,4-dimethyl cholest-4,6,8(14)-triene and cycloartenyl acetate, Table 1). Taking into account that the biological potential of German chamomile and its value as anti-inflammatory and anti-spasmodic medicinal plant are dependent on matricine (as precursor of chamazulene) and α-bisabolol and bisabolol oxides content, the separation of essential oil from unwanted components under the conditions that would not cause any valuable compounds degrading might be achieved by applying certain $ScCO_2$ conditions. Further investigation might be directed to obtain the extract rich in matricine, because of the advantage of low temperature regime $ScCO_2$ takes place. Considering the features of applied extraction method, the separation of fatty acids and wax (almost 40% of the analyzed extract), might represent the aim of further experiments in order to successfully perform the purification of desired fraction.

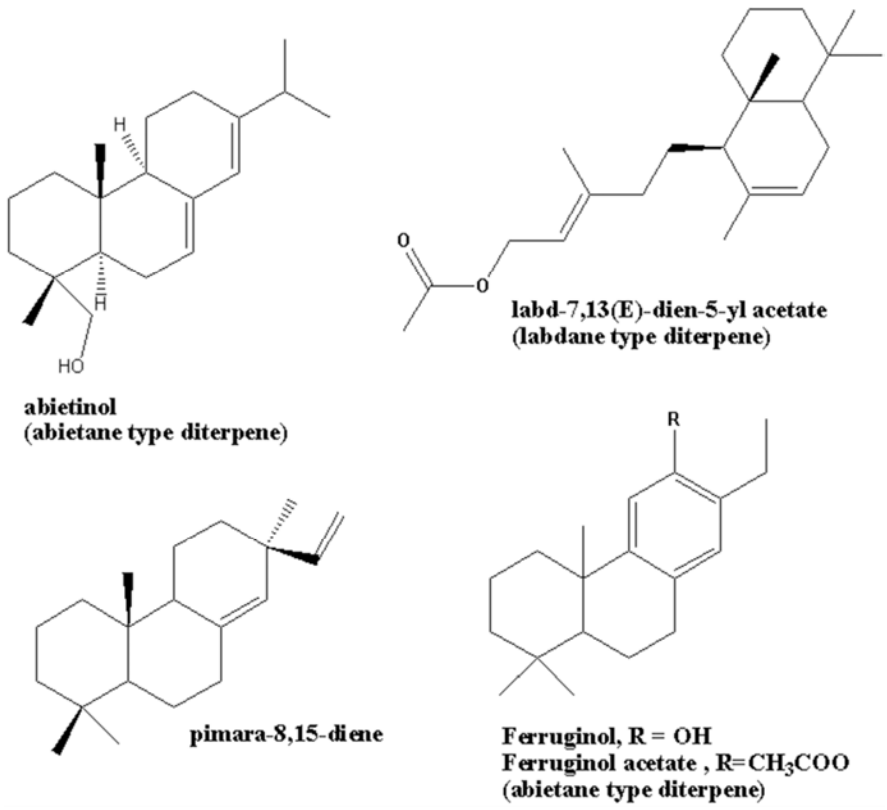


Fig. 2. Some of identified diterpenes present in investigated extracts (given the basic structures whose derivatives have been identified applying GC techniques)

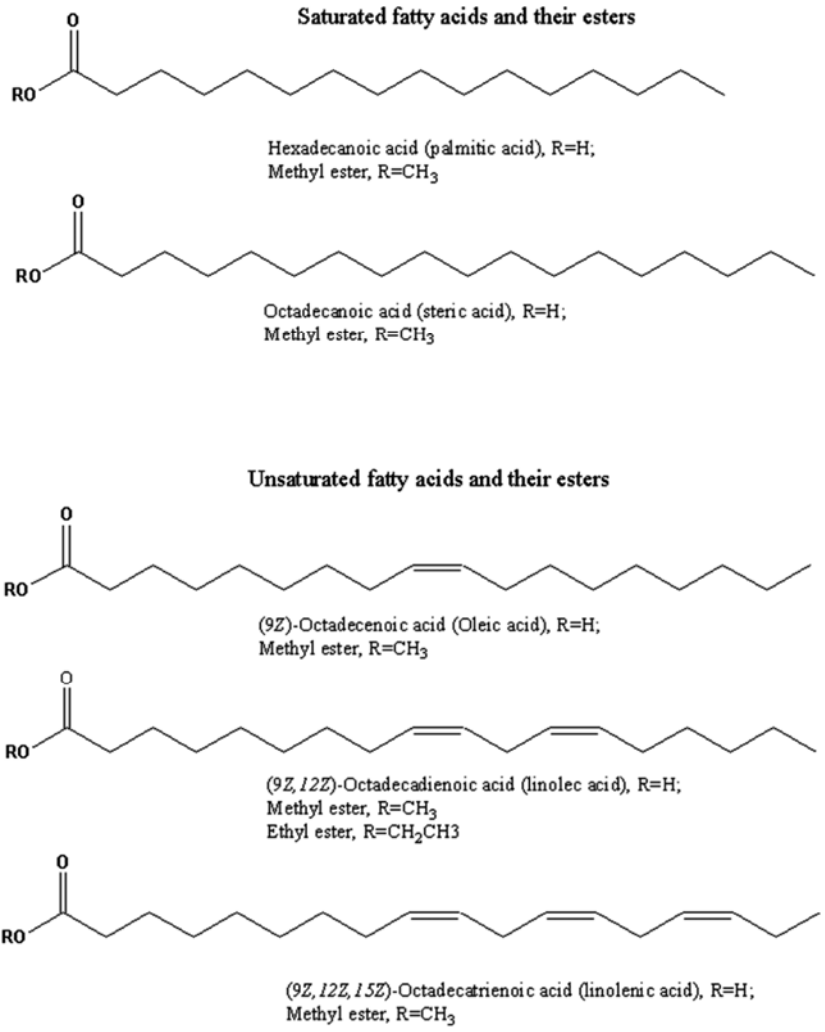


Fig. 3. Some of the representatives of saturated and unsaturated fatty acids and their esters identified in investigated extracts

Black caraway, black cumin (*N. sativa*) seeds have been used for thousands of years as a spice and food preservative. Black cumin is an annual herbaceous plant widely grown in the Mediterranean countries, Middle East, Eastern Europe and Western Asia. The seeds have been added as a spice to a variety of Persian foods such as bread, yogurt, pickles, sauces and salads. In the Middle East, Northern Africa and India, it has been used traditionally for centuries for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema and for its antihistaminic, antidiabetic and anti-inflammatory activities. The oil and the seed constituents, in particular thymoquinone, have shown potential medicinal properties; they exhibit potent anti-inflammatory

effects on several inflammation-based models including experimental encephalomyelitis, colitis, peritonitis, oedama, and arthritis through suppression of the inflammatory mediators prostaglandins and leukotriens. The oil and active ingredient thymoquinone showed beneficial immunomodulatory properties, augmenting the T cell and natural killer cell-mediated immune responses. Most importantly, both the oil and its active ingredients expressed anti-microbial and anti-tumor properties toward different microbes and cancers. Coupling these beneficial effects with its use in folk medicine, black cumin seed is a promising source for active ingredients that would be with potential therapeutic modalities in different clinical settings. The effects of main components present in black cumin, quinones thymoquinone, dihydrothymoquinon, and phenolic compounds thymol and carvacrol, were investigated regarding beneficial effects in different central nervous system disorders including memory impairment, epilepsy, and neurotoxicity. The literature data revealed that *N. sativa* seed and mentioned main constituents exhibited their activity through the inhibition of AChE activity and increasing the GABAergic tone and particularly significant antioxidant effects [31–33].

Our results indicated the abundant presence of hexadecanoic and 9*Z*,12Z-octadecadienoic acids (22 and 66.7%, respectively), making almost 90% of the components identified in the investigated $ScCO_2$ extracts. In addition, the analysis revealed the presence of diterpenes pimara-8,15-diene and phytol (Figures 1, 2, 3, 4; Table 1). The identified compounds with the already mentioned biological properties might be recognised as substances contributing or enriching the traditionally known application. The conclusion regarding the application of $ScCO_2$ extraction in a case of this medicinal plant might be that by controlled extraction, certain fractions might be obtained being rich in specific compounds, not limited only to quinones.

The performed GC analysis of the investigated stinging nettle $ScCO_2$ extract revealed interesting composition regarding non-volatile composition – pigment components were not detected, but the extracts was rich in phytol, a diterpene alcohol from chlorophyll (22.5%) and sterol components (6.7%), with β-sitosterol being most abundant. Linoleic and palmitic acids were present in significant amounts (8.5 and 4.2%, respectively) (Figures 1, 3, 4, 5B; Table 1). Phytol has been widely used as a food additive and has medicinal application, possessing the promising antischistosomal properties *in vitro* and antinociceptive activity [34–37]. The European Foods Safety Authority (EFSA) recommends consuming about 1.5–2.4 g/day of phytosterols in order to reduce blood cholesterol. Moreover, phytosterols, structurally similar to cholesterol and existing in several forms in plants including β-sitosterol, campesterol, stigmasterol and cycloartenol, could be incorporated in diet not only to lower the cardiovascular disease risk, but also to potentially prevent cancer development [38]. Of these, sitosterol is the most abundant phytosterol, followed by campesterol. The presence of β-sitosterol among them especially, might be of imortance, having in a mind conducted research regarding β-sitosterol numerous activities, stressing its anti-inflammatory potential [39, 40].

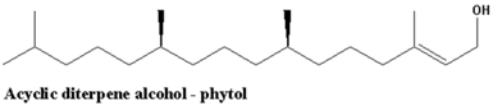


Fig. 4. Acyclic diterpene alcohol in investigated extracts

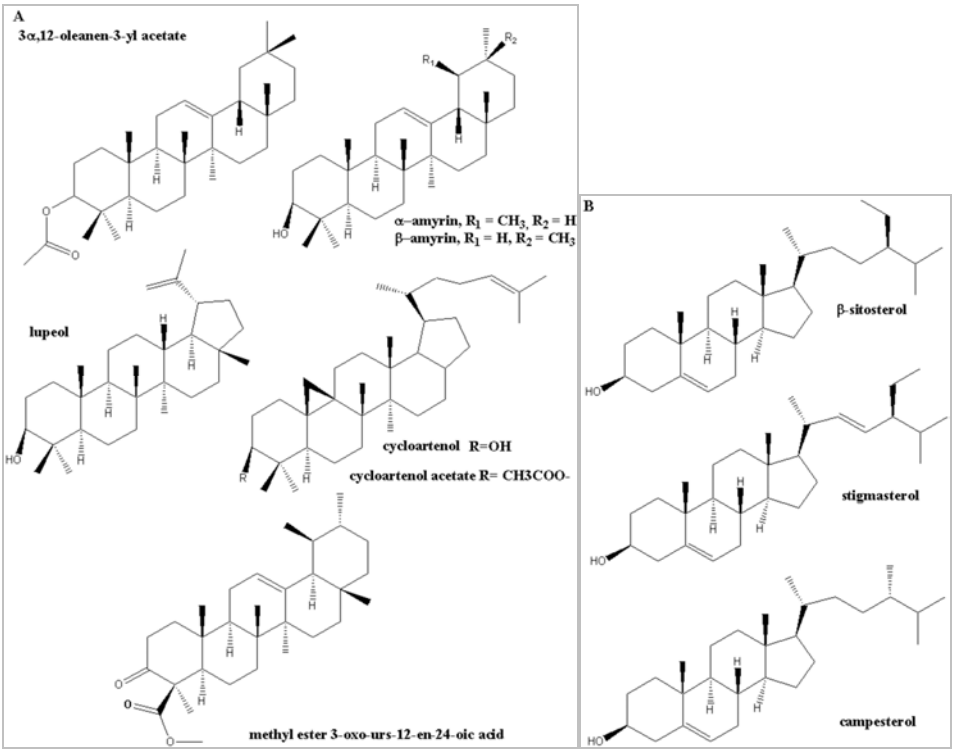


Fig. 5. Some of identified triterpenes (A) and phytosterols (B) present in investigated e xtracts

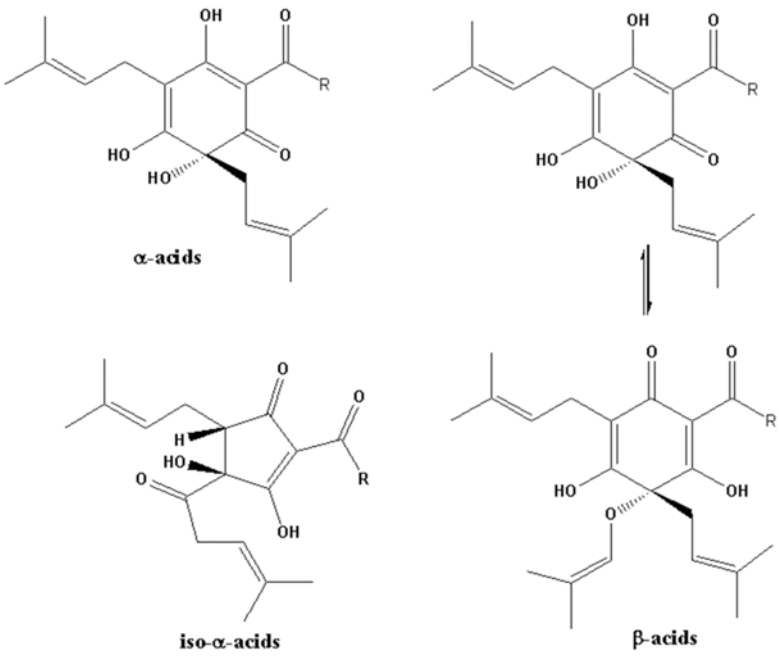
Since ancient times, hops (*H. lupulus*) have been used in traditional medicine for their claimed anti-inflammatory, antiseptic, antidiuretic, aphrodisiac, hypnotic, sedative, and stomachic properties. The German Commission E approved a monograph on hops for use in mood and sleep disturbances. Similar indications are described in an ESCOP (European Scientific Cooperative on Phytotherapy) monograph. Increasing evidence reveals that the so-called hop bitter acids, which represent up to 30% of the total lupulin content of hops, prenylflavonoids and xanthohumol exhibit interesting effects on human health. According to more or less pronounced bioactive effects associated with practically all hops secondary metabolites, the pharmaceutical industry has considered it as a potential source of new plant derived medicines [9, 41].

The chemical analysis of hop $ScCO_2$ extract revealed the presence in significant quantity all secondary metabolites whose presence in this paper were

monitored, namely phloroglucinol derivatives, steroids and fatty acids and their derivatives (24.8, 10.7 and 9.7%, respectively) (Figures 1, 3, 5, 6; Table 1). In the investigated extract, besides the volatile components, which were detected in quantities corresponding to literature data [42], the non-volatile, the most active phloroglucinol derivatives were determined (Figure 6), humulone derivatives prevailed in comparison to β-acids, lupulone (18.2 and 8.6%, retrospectively).

Many studies have been performed on chemical composition in regard of the phloroglucinol derivatives – bitter acids content in differently prepared hop extracts, and extracts that undergo the chemical changes, resulting in isomerization of α-acids, humulones into corresponding isomers. Besides the influence on olfactory properties of beer, hop bitter acids have interesting and various pharmacological properties. Namely, antioxidative activity of hop bitter acids and their analogues may contribute to a cancer preventive effect since they can quench free radicals which cause oxidation of the DNA in the body and thus causing genetic defects. Humulon has been known for its preventive effect against osteoporosis. Humulon hinders the growth of certain leukemia cells especially with the combination of vitamine D. The results of one recent study confirmed that beer components are protective against the genotoxic effects of heterocyclic amines on target organs associated with tumorigenesis „in vivo“. Isomerized bitter acids (isohumulones) might prevent the developments of noninsulin dependent diabetes and hyperlipidemia, improving insulin sensitivity in patients with type 2 diabetes. In addition, the preservative properties of hop have been investigated for many years, considering the bitter acids as the main antibactetial compounds. Recent investigation of lupulone activity in colon cancer chemoprevention trials, demnostrated that this phloroglucinol derivative is able to activate TRAIL-death signalling pathways even in TRAIL resistant cancer cells, what highlights the potential of this natural compound for cancer prevention and therapy [9, 43].

Two $ScCO_2$ extracts of hemp (*C. sativa*) were analysed, and the results were shown at Figures 1, 2, 3, 4, 7; Table 1. The purpose was to give evidence in chemical profile of extracts obtained by $ScCO_2$ extraction, which significantly differenced in term of cannabinoids content. Namely, hemp extract 1 was characterized by significant presence of 9,12-octadecadienoic acid and cannabidiol (53 and 6.6 %, respectively), while hemp extract 2, purified extract contained ~67% of cannabidiol (CBD), with the other cannabinoids derivatives, like cannabichromene, cannabicitran, cannabigerol, cannabielsoin and cannabinol, present in traces. 9,12-Octadecanoic acid was not detected in hemp extract 2. The presence of valuable compound methyl ester of 9,12,15-octadecatrienoic acid, so rarely present in food, was confirmed in extract 1 (1.9%). In both samples the presence of methylated phenols (vitamin E), sterols and triterpenes (β-sitosterol, β-stigmasterol, β-amyrin, α-amyrin, 3α-12-oleanen-3-yl acetate, cycloartenol and its acetate, methyl ester of 3-oxo-urs-12-en-24-oic acid, lupeol acetate) were detected in traces.



α-acids (humulones)			β-acids (lupulones)
1	(n)-:	$R = CH_2CH(CH3)_2$	6
2	co-:	$R = CH(CH_3)_3$	7
3	ad-:	$R = CH(CH)_3CH_2CH_3$	8
4	pre-:	$R = CH_2CH_2CH(CH_3)_2$	9
5	post-:	$R = CH_2CH_3$	10

Fig. 6. The main phloroglucoinol constituents of *Humulus lupulus L.,* Cannabaceae *(*hop*)*

Nowadays, we are aware of renewed interest in hemp's extracts and their activity, of continuous effort to establish the extraction condition that would enable extraction of non-psychotropic components. The main active compounds present in *C. sativa* were cannabinoids (Figure 7), which modulate a series of important physiological functions ranging from locomotors activity to memory, from pain perception to food intake, from inflammatory reaction to cancer development. To date, two cannabinoid receptors have been characterized: 1) the cannabinoid receptor 1 (CB1) primarily expressed in tissues of the CNS and 2) the cannabinoid receptor 2 (CB2) mainly expressed in the immune system. The CB1 is a prime target for the psychoactive effect of cannabinoids, whereas cannabinoid induced immunomodulation is predominantly CB2-mediated. Considering that inflammation plays an important role in the pathology of most neurodegenerative diseases, the ability to control the degree of inflammatory reaction would be advantageous. The potential participation of the CB2 receptor

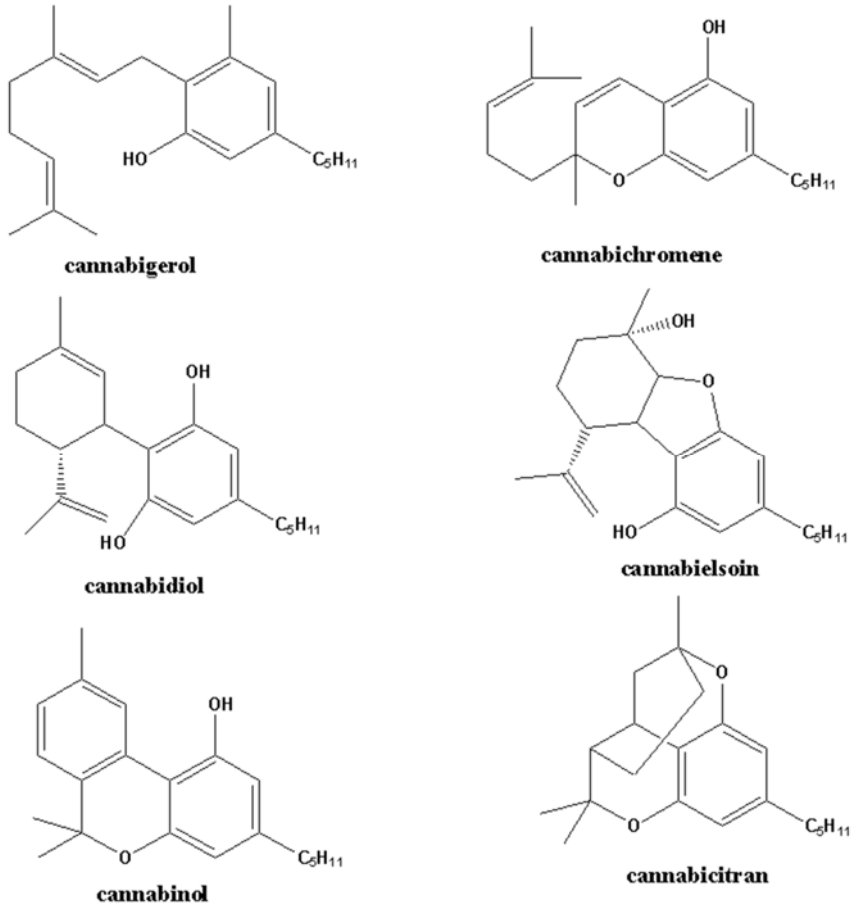


Fig. 7. The main classes of cannabinoids identified in investigated extra/ct of *C. sativa*

in this process may represent a mechanism by which therapeutic intervention could be achieved. The CB2 receptor is abundant in immune tissues, in particular in spleen macrophages and in tonsils. Interacting with most cells of the immune system, natural and synthetic cannabinoids are able to modulate multiple immune responses, both in humans and rodents. Cannabinoids can inhibit T-lymphocyte functions such as proliferation and cytotoxicity, decrease antibody formation by B cells,and affect production of several cytokines, mostly decreasing interleukin-2 (IL-2), interferon-χ (IFN-χ), and IL-12 and increasing the levels of IL-4 and IL-10. Cannabinoids could exert an effect on macrophage/microglial cells by modulating acute-phase cytokine release, mainly IL-1, tumor necrosis factor-α (TNF-α), and IL-6, which play a major role in the development of damage in neurodegenerative/neuroinflammatory conditions, such as cerebral ischemia. Also, as there is a body of experimental evidence of

the activity by cannabinoids that cannot be explained by their interaction with receptors, these data suggest the existence of other mechanisms involved in cannabinoid effects. The two most investigated receptor-independent mechanisms of cannabinoids are their antioxidant properties and their capacity to alter cell-membrane properties. Despite the emerging evidence regarding therapeutic activities of CBs, their effective introduction in clinical use is still controversial and strongly limited by the unavoidable psychotropic effects exhibited by many of them. Up to the present, the molecular pharmacology of one of the most interesting canabinoids, CBD has not yet been well defined and little is known about a possible CBD-receptor-mediated signaling pathway. CBD binds with a low affinity to both CB1 and CB2 cannabinoid receptors; hence CB1 and CB2 independent modes of action for this phytocannabinoid have been recognized. CBD can bind to sites different from cannabinoid, stimulating the type-1 vanilloid receptor. CBD also binds to 5-HT1A and such an interaction was suggested to account for the attenuation of cerebral infarction size occurring during ischemia and also for its anxiolytic effect. Although CBD displays very low affinity for both CB1 and CB2, it has been reported recently that it can operate as a CB2 receptor inverse agonist and this may, at least in part, contribute to its widely documented its antiinflammatory properties. Furthermore it is commonly recognized that many CBD effects are mainly due to its antioxidant activity. Recent investigations have demonstrated, to various degrees, antibacterial activity for non-psychotropic cannabinoids cannabidiol, cannabichromene, cannabigerol and pre-cannabidiol [44, 45].

3. SUMMARY

The aim of supercritical extraction of different plants is mainly focused to optimize extracts isolation with specific composition of active components, gaining attention as an advanced procedure to increase the recovery of bioactive compounds from natural matrices. Applications of ScFE include analytical-scale extractions and screening, as well as pilot-scale extraction. Some products possibly produced by ScF technology may be found on our everyday table. Examples of such products are vitamin additives, dealcoholised beverages, non-fat potato chips, and encapsulated liquids. But, nowadays, the use of ScFE in natural product chemistry overlaps the fast growing area of so-called nutraceuticals, food products or additives intended to treat or prevent disease.

The $ScCO_2$ extraction represents the attractive alternative to conventional extraction and refining methods for the food processing and pharmaceutical industries. Although the technique struggles with the cost-effectiveness for low volume products, it is overcoming this limitation more effectively everyday and providing economic benefits for larger volume products as well. Overall yield of extract can be different depending on extraction conditions. However, not only the yield of different fractions can be affected by choosing specific pressure and

temperature used in a process of supercritical extraction but in many cases some desired characteristics of obtained extracts could be achieved. The optimal extraction conditions thus depend on the purpose and further use of prepared extracts [46-49].

Taking into account the lipophylic properties of terpenes, fatty acids, phloroglucinol and orcinol derivatives, $ScCO_2$ extraction has received attention as an alternative to organic solvent extraction. In theory, each compound possesses a unique extractability under different conditions of supercritical fluid related to factors such as extraction temperature, pressure, and time. Thus, components in a sample are extracted in an ordered manner from a sample matrix under optimized conditions of SFE, allowing the convenient fractionation of the extract. This can reduce the cost and time involved in the separation compared to traditional solvent extraction for purifying specific components of interest from the extract [50].

Substantial attention has been given to primary cancer prevention in daily life, where different types of terpenoid compounds have been finding the irreplaceable position. It is known that dietary factors are through to contribute to as much as one-third of the factors influencing the development of cancer [51]. Some of the components of a plant-based diet are phytosterols, like β-sitosterol and taraxasterol, with numerous biological activities, anti-inflammatory, cholesterol-lowering, anti-microbial, anti-bacterial, anti-fungal effects. Apart from the mentioned activities, their anti-tumour and chemopreventive potential have been attracting the attention. They have been shown experimentally to inhibit colon and breast cancer development. They act at various stages of tumour development, including inhibition of tumorigenesis, inhibition of tumour promotion, and induction of cell differentiation. They effectively inhibit invasion of tumour cells and metastasis. With regard to toxicity, no obvious side effects of phytosterols have been observed in studies to date, with the exception of individuals with phytosterolemia. Several extracts that were investigated in this work showed the significant presence of phytosterols - *U. dioica* ScCO2 extract being the richest.

Fat is a crucial macronutrient that affects the development of cardiovascular disease (CVD), which in turn is the most prevalent complication of obesity. The recommended nutritional strategy for CVD prevention is increasing consumption of unsaturated fatty acids, including PUFAs, at the expense of saturated fatty acids (SFAs). Numerous mechanisms whereby dietary PUFAs can ameliorate obesity-related disorders and reduce CVD risk have been identified, including beneficial effects on adipocyte-derived hormones, a low-grade inflammation, blood lipids, blood pressure, and vasodilation. $ScCO_2$ extracts of *M. officinalis, N. sativa* and *C. sativa* were interesting because of significant presence of the fatty acids.

Although primarily used today as one of the most prevalent illicit leisure drugs, the use of *C. sativa* L., commonly referred to as marijuana, for medicinal purposes has been reported for more than 5000 years. Marijuana use has been

shown to create numerous health problems, and, consequently, the expanding use beyond medical purposes into recreational use (abuse) resulted in control of the drug through international treaties. In this context, much attention has been focused on the main non-psychotropic component of the glandular hairs of *C. sativa* cannabidiol (CBD) that constitutes up to 40% of the *Cannabis* extract, and represents one of the most promising candidates for clinical utilization due to its remarkable lack of any cognitive and psychoactive actions. CBD is regarded as an interesting putative lead compound to develop cannabinomimetic drugs, because of its excellent tolerability in humans. Application of $ScCO_2$ extraction, under controlled temperature and pressure might enable obtaining extract rich in CBD, while at the same time the undesirable conversion of cannabinoids into psychotropic substance THC might be avoided.

Hop extracts have long been used in traditional medicine mainly for the treatment of the sleeping disorders, and for the activation of impaired gastric function. Also, it is well-known as the raw material in the brewing industry. The presented results of this investigation confirmed the possibility to apply the $ScCO_2$ extraction for production of hop extracts rich in acyl phloroglucinol derivatives, as compounds with significant anti-oxidative properties.

The investigated extracts provided valuable information regarding the possible application and definition of the specific conditions applied in $ScCO_2$ extraction in order to provide extracts rich in specific biologically active components. Irrespective of whether herbs are used for medicines, cosmetics or foods, they must be safe, of standard quality and efficient. In order to ensure a high level of protection for consumers and facilitate their choice, the products that will be put on to the market have to be safe and bear adequate and appropriate labelling. In that light, the quality of phytomedicine preparations will be defined by the quality of the herbal drugs, implemented extracts, by the process of manufacturing of the drug preparations and the properties of the finished product, taking into consideration the requirements in accordance with the or ISO or HACCP or Good Manufacturing Practice (GMP) standards [52, 23].The clean technology of $ScCO_2$ might provide the complete fulfilment of the requested regulative for the production of the extracts with defined and standardized composition, rich in components with the significant biological activities.

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APPLICATION OF SUPERCRITICAL FLUIDS IN DEVELOPMENT OF MATERIALS WITH ANTIBACTERIAL PROPERTIES

Summary

In this chapter examples of development materials with antibacterial properties applying batch supercritical solvent impregnation are presented. Natural active components and plant extracts gained special attention as antibacterial agents due to their efficiency and passive role referred to the further bacterial resistance. Thymol and carvacrol were selected as model compounds. Results on impregnation of cotton gauze, polypropylene non-woven material and cellulose acetate with thymol and/or carvacrol were reviewed with respect to processing conditions, yield obtained and antibacterial activity of the impregnated materials. New data on the impregnation of cellulose acetate with carvacrol as well as foaming and impregnation of poly(lactic acid)-based biomaterials and starch based films with thymol were presented. Brief summary results on the combined supercritical fluid extraction – supercritical solvent impregnation process for impregnation of plant extracts is provided.

1. INTRODUCTION

1A. Scope of research

This chapter is aimed to stress the great potential of supercritical fluid application in development of materials with antibacterial properties. High pressure impregnation using supercritical carbon dioxide ($scCO_2$) is a powerful tool for fabrication of sophisticated added value polymer-based materials. Demand for polymer-based materials with antibacterial properties is of increasing interest nowadays due to the one of the greatest threats of the modern world – bacterial resistance. As we are aware – resistant bacterial strains emerged in hospitals and animal farms and spread to community expressing zoonotic potential (spreading from humans to animals and otherwise) [1]. World

Health Organization announced the pandemic of panresistance, meaning that strains resistant to all existing antibiotics were found in each country and in every continent [1]. Besides plenty of data in scientific journals, leading world daily journals have started to point out severity of the situation. On May 19, 2016 the INDEPENDENT published an article entitled "Antibiotics will stop working at a 'terrible human cost', major report warns" presenting data from the report prepared for the UK Government. The report is the result of a two year long review of the use of antibiotics and was commissioned by the Government amid growing concerns about the use of the medicines in the UK. According to the report: "It will soon be too dangerous to perform caesarean sections, joint replacements and chemotherapy if antibiotics become ineffective." and "Unless urgent action is taken, drug resistant infections will kill 10 million people a year by 2050, more than cancer kills currently".

Plant extracts and purified natural components may have strong antibacterial activity even against resistant strains [1]. Additionally, very important advantage of plant extracts application as antibacterial agents is the fact that plant extracts do not contribute to the further resistance. Considerable solubility of secondary plant metabolites (e.g. essential oils as carriers of antibacterial activity) in $scCO_2$ enables development of materials with antibacterial properties by supercritical solvent impregnation (SSI) process.

In this chapter, examples of development of materials with antibacterial properties using SSI from the Laboratory for the high pressures at the University of Belgrade Faculty of Technology and Metallurgy will be presented. As model natural components with strong antibacterial activity thymol and carvacrol were used.

2. MAIN PART

The SSI experiments with thymol and carvacrol as natural bioactive substances were carried out as batch processes in a high pressure vessel or a high pressure view chamber as described in the literature [2] and [3] respectively. For this purpose thymol (>99%, Sigma Aldrich, Germany), carvacrol (≥98%, Sigma Aldrich, Germany) and commercial carbon dioxide (purity 99%, Messer-Tehnogas, Serbia) were used.

2.1. Impregnation of cotton gauze

Woven gauze is the oldest wound dressing still in use and the first written evidence on its usage dates from the time of Ancient Egypt. The Ancient Egyptians used it for wound treatment as well as to wrap bodies prior to burial [4]. Today, woven gauze is seen as a 'wet to dry' dressing and despite its non-selective mode of physical debridement, possible trauma to the wound bed and resultant pain, it is still the most utilized wound dressing in the world which can be also utilized as a vehicle for antimicrobial agents [5].

In order to produce modified cotton gauze with antibacterial properties, a study on SSI with thymol was performed [2]. Temperature of 35°C and pressure of 15.5 MPa were selected as operating conditions for the SSI on the basis of the solubility data analysis of thymol in $scCO_2$. Process time was varied from 2 h to 24 h. Impregnation yield obtained after 2 h of impregnation was 11%, while the value of 19.6% was reached after 24 h. FT-IR analysis confirmed the presence of thymol on the surface of the cotton fibers. The impregnated gauze (11% and 19.6%) provided strong antimicrobial activity against tested strains of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *E. faecalis* and *Candida albicans.*

Impregnations of cotton gauze with carvacrol were carried out at temperature of 50°C and pressures of 10, 21 and 30 MPa whereby impregnation yields obtained varied from 4% – 14.2% [6]. Activity of the cotton gauze impregnated with carvacrol was tested against *S. aureus* and *E. coli* strains. The results of antibacterial analysis showed that strong antibacterial activity against both strains was obtained when the impregnation yield was 12.4% or higher. This corresponds to processing times of 12.5 h at 30 MPa and 24 h at 21 MPa [6].

The results obtained indicated high potential of SSI for production of gauze with antibacterial properties. This way fabricated cotton gauze can be used as a secondary wound dressing to prevent wound contamination. In order to apply it as a primary wound dressing more data on cytotoxic activity of the material are needed.

2.2. Impregnation of cellulose acetate

Cellulose acetate (CA) is a thermoplastic material produced by the esterification of cellulose in the reaction with acetic anhydride and acetic acid in the presence of sulfuric acid. CA is a biodegradable and biocompatible polymer. Depending on its processing parameters it is suitable for different applications: immobilization of enzymes, as a medium for separation, specific adsorbent, for controlled release of active pharmaceutical ingredients, or as a medical gauze, ribbon, lining, home furnishing [7, 8]. There are literature reports on impregnation of CA with different components using $scCO_2$. A near-critical and $scCO_2$- assisted impregnation process has been proposed to load vanillin and L-menthol into CA fibers without disruption of the fiber morphology, taking advantage of the polarity of these two compounds and of their hydroxyl groups that were suitable hydrogen bonding sites [9]. Impregnation with thyme extract (*Thymus vulgaris*), using integrated process of supercritical extraction of thyme and impregnation of CA, for possible application in food industry was also reported [10] whereby impregnation yield of 1.4% was obtained. Milovanovic and coworkers [3] investigated impregnation of CA with thymol whereby CA (32% acetyl content, Eastman, Poland) was used. High loading capacity (maximal impregnation yield) of 72% was determined indicating hydrogen

bonding of thymol and CA chains. The loading capacity was found not to be dependent on the processing conditions (pressure and temperature), while the rate of impregnation was faster at higher pressures. Our group investigated impregnation of CA with carvacrol as well. In the case of carvacrol, loading capacity of CA was determined to be 62.5%. In Fig. 1 kinetics of impregnation of CA with thymol and carvacrol at selected SSI conditions (20 MPa and 35°C for thymol and 21 MPa and 50°C for carvacrol) are presented.

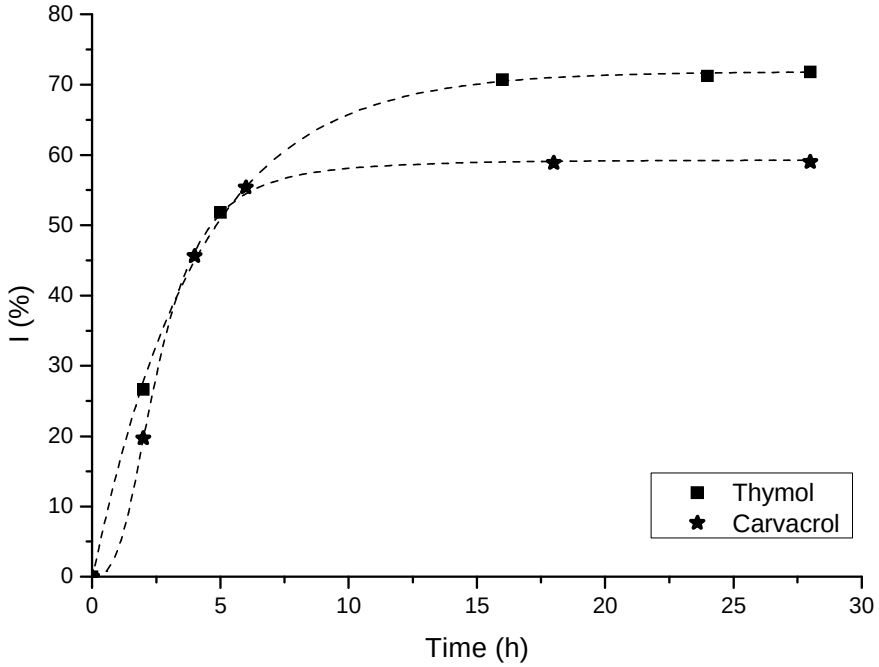

Fig. 1. Impregnation yield (I (%)) as a function of time for thymol (10 MPa and 35°C) and carvacrol (10 MPa and 50°C) impregnation

Solubility of carvacrol in $scCO_2$ [11] is higher than the solubility of thymol [2] at lower pressures (e.g. 10 MPa), which yielded in the faster impregnation of CA with carvacrol at 10 MPa. In Fig. 2 comparison of obtained yields for thymol and carvacrol at 10 MPa was presented.

Images of the impregnated CA recorded by a field emission scanning electron microscope (FE-SEM, Mira3, Tescan) showed that higher impregnation yields of thymol and carvacrol contributed to change in CA morphology from swelling (for impregnation yields higher than 9%) to agglutination (for impregnation yields higher than 55%). Fig. 3 shows that lower content of thymol and carvacrol in CA (4.5% and 6.0%, respectively) didn't affect the CA morphology.

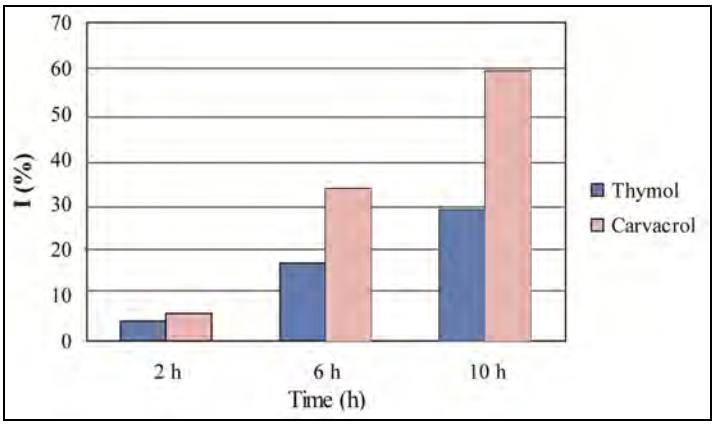

Fig. 2. Impregnation yields obtained at 10 MPa for the SSI of carvacrol (at 50 °C) and thymol (at 35°C)

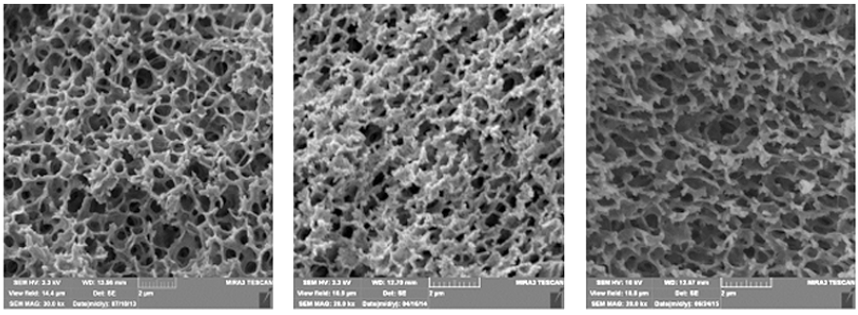

Fig. 3. Morphology of cellulose acetate a) non-treated, b) impregnated with thymol 4.5% and c) impregnated with carvacrol 6.0% (scale bar = 2 μm)

Results of antibacterial activity showed that even lower impregnation yields for thymol and carvacrol achieved after 2 h of impregnation (4.5% and 6.0%, respectively) were sufficient to ensure antimicrobial activity of the CA samples, while samples with impregnation yield around 30% showed very strong antibacterial activity against tested *S. aureus*, *E. coli*, *Salmonella enteritidis* and *B. cereus* strains.

The results presented indicate high potential of SSI in production of cellulose acetate based materials with antibacterial properties offering wide rang of active substance content – in case of thymol and carvacrol from 4% to 72% and 62.5% respectively. The wide range of active substance content facilitates wide range of release time – in the case of thymol up to 21 days [3].

2.3. Impregnation of poly(lactic acid)-based biomaterials

Poly(lactic acid) or polylactide (PLA) stands for the century's most promising green material with 35% contribution to the bioplastic market [12]. It is an aliphatic thermoplastic, high-strength, high-modulus polyester obtained by biochemical synthesis from renewable feedstock (starch and sugar) by the ring-opening polymerization of lactide, the cyclic dimer of lactic acid [13]. Lactic acid exists in optically active D-or L-enantiomers which ratio in PLA determines polymer crystallinity and, consequently, its permeability, mechanical performance, heat deflection temperature and biodegradation rate [14, 15]. This allows production of a wide spectrum of PLA polymers to match specific performance requirements. Given the reasonably good optical, physical, mechanical, and barrier properties PLA can compete with petroleum-based thermoplastic polymers for numerous applications. PLA has been approved by the US Food and Drug Administration (FDA) for food and pharmaceutical applications. Until the last decade, its use was limited to medical applications such as implant devices, tissue scaffolds, and internal sutures due to its high cost, low availability and limited molecular weight. Modern polymerization techniques have enabled economical production of high molecular weight PLA (greater than 100,000 Da) and broadened its use for food and cosmetics packaging, textile, car interiors, agricultural sheets, household appliances, etc. [15].

A great potential of SSI for fabrication of advanced PLA materials for both biomedical applications and consumer's goods has been justified in the recent studies. Namely, SSI has been successfully applied for loading of bioactive substances into the non-toxic and bioresorbable PLA-based stents at physiologic temperatures [16]. Micell Technologies (Durham, NC, USA) employs supercritical fluid technology to impregnate poly(lactic-co-glycolic acid) (PLGA) coating of the modern drug-eluting stents with anti-inflammatory and anti-restenotic drugs (sirolimus) to provide an uniform drug delivery for the entire polymer absorption period (after 90 days) and its presence for months after polymer absorption [17]. Commercial potential of PLA for biodegradable and active packaging has been recently recognized [18, 19]. Batch SSI was successfully employed for loading a high amount of anti-inflammatory drugs, ketoprofen (32%) and aspirin (8%), into commercial PLA suture fibres at CO_2 pressure of 35 MPa and 80 °C after 3 h without adverse effect on elastic properties of the substrate [20]. Supercritical fluid technology was applied for one-step creation and impregnation of PLA and PLGA scaffolds with anti-inflammatory and anticancer drugs (indomethacin [21] and 5-fluorouracil [22]) using $scCO_2$ at moderate pressure and temperature conditions (18 MPa and 40°C) within an hour. In the aforementioned studies, stirring and depressurization rate were optimized in regard to drug loading and release profile to justify potential of the scaffolds for tissue engineering and controlled drug delivery applications [21, 22]. Recent study on PLA fibres impregantion

with tranilast using $scCO_2$ at 22 MPa and 80–100°C with 6–10 vol% EtOH showed SSI potential for an antiallergenic textile production. Impregnation yields of tranilast achieved after an hour at the aforementioned SSI conditions were 0.3–0.5% based on the weight of fibre. However, there is a scarce data on utilization of SSI for loading natural bioactive substances into PLA films and scaffolds [23, 24].

In this chapter, feasibility of loading thymol and thyme extract into commercial medical, extrusion and injection grade PLA and Poly(lactic-*co*-glycolic) acid (PLGA) samples using $scCO_2$-assisted processes with different flow regimes is presented. Medical grade amorphous Purasorb polymers, PLA and PLGA (PDL 02 and PDLG 7502, respectively) obtained from Corbion Purac Biomaterials (Netherlands) with glass transition temperature (T_g) similar to physiologic temperature were used for the batch SSI with thymol. Semi-crystalline PLA samples Ingeo biopolymer (grade 2003D, NatureWorks LLA, Nebraska, USA) were used for the batch SSI with thymol in static (s-SSI) and dynamic mode (d-SSI). The latter implied circulation of the supercritical solution. The same polymer was used in combined supercritical fluid extraction (SFE)-SSI process (see part 2.6) to load extract of thyme (*Thymus vulgaris* L). Ingeo bioplolymer (grade 3052D, NatureWorks LLA, Nebraska, USA) was used to prepare film for a batch SSI impregnation with thymol for potential use in active food packaging. Film of PLA (thickness of 0.2 mm) was obtained by solvent casting method using chloroform as a solvent (concentration of PLA = 0.08 g/mL). Static SSI was performed in a 25 mL high pressure view cell (Eurotechnica GmbH, Bargteheide, Germany). Dynamic SSI and SFE-SSI were carried out in the HPEA 500 (Eurotechnica GmbH, Bargteheide, Germany) unit [10] consisted of a high pressure extraction vessel (280 mL) and adsorption vessel (100 mL). Mass of the PLA in all the experiments was 0.2 g. Thymol to PLA mass ratio was 1:1 (s-SSI) and 7.6:1 (d-SSI). The higher thymol amount in d-SSI was needed in order to be achieved the same saturation of the supercritical fluid as in the s-SSI process. Mass of the thyme used for the SFE-SSI process was 25 g. Impregnation yields at optimized process conditions are presented in Table 1.

Table 1. Loadings of thymol and thyme extract at optimized process conditions

Substrate	Impregnation substance	Process	P (MPa)/T (°C)/t (h)	Impregnation yield (%)
PLGA (Purasorb)	Thymol	s-SSI	7.5/35/2	3.0
PLA (Purasorb)	Thymol	s-SSI	10/40/4	4.9
PLA (Ingeo)	Thymol	s-SSI	30/100/2	5.0
PLA (Ingeo)	Thymol	d-SSI	30/110/2+5[a]	1.1
PLA (Ingeo)	Thyme extract	SFE-SSI	30/110/2+2[b]	1.2
PLA (Ingeo)-film	Thymol	s-SSI	10/40/2	6.6

[a]2 h of static and 5 h of dynamic regime;

[b]2 h of dynamic regime with venting out of adsorber and introduction of fresh CO_2.

All the foams obtained by the fast depressurization upon soaking in $scCO_2$ or $scCO_2$+impregnating substance had micro-sized pores (several to 100 μm). Maximum decompression rate achieved in the d-SSI and SFE-SSI processes (3.8 MPa/min) was a bit lower compared to s-SSI process (3 MPa/min). Foams produced from amorphous PLA and PLGA had open cell structure with interconnected pores (Fig. 4). Loading of 3–5% of thymol into amorhous PLA and PLGA resulted in formation of foams with smaller pores and thicker cell walls. Dissolution of $scCO_2$ induces crystallization of amorphous polymers and promotes crystallization of semi-crystalline polymers [25–27]. This is due to the increased macromolecular chain mobility in the presence of gas molecules which favours their folding and formation of crystalline lamellae. Presence of thymol additionaly plasticize polymer substrate [3, 28] which can affect form of crystallites and their arragments into thicker lammelas [26].

Pore size and interconnectivity is crucial for end-application of polymer foams in biomedicine [29]. Earlier studies demonstrated optimal pore size of 5 μm for fluid diffusion and neovascularization, 5–15 μm for fibroblast ingrowth, 20 μm for ingrowth of hepatocytes, <50 μm for cartilage ingrowth, 20–125 μm for regeneration of adult mammalian skin and 200–350 μm for osteoconduction [29, 30]. Porosity and interconnectivity is vital for artificial blood vessels or peripheral nerve growth and fluid uptake [29, 31]. In this regard, obtained PLGA and PLA foams loaded with thymol (Fig. 4b and 4c) with an interconnected pore structure and cell-size of several to ~50 μm have a potential for controlled drug delivery and soft tissue engineering applications.

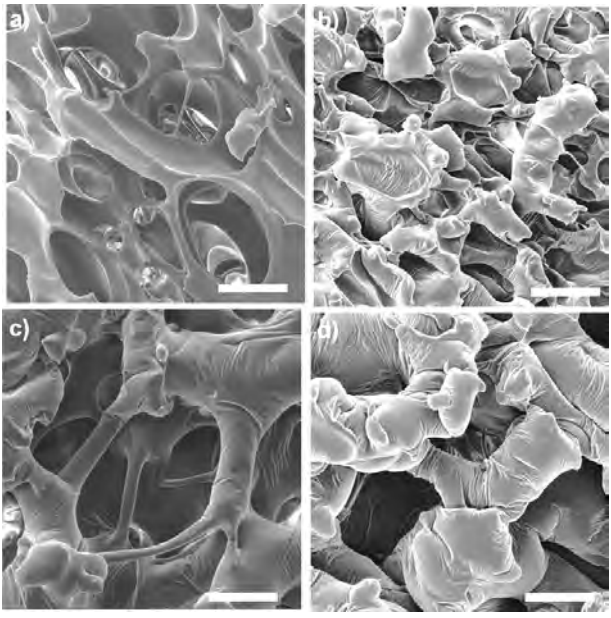

Fig. 4. Morphology of (a) neat and (b) impregnated PLGA foam, (c) neat and (d) impregnated PLA foam (scale bar = 50 μm)

Impregnation and foaming of semi-crystalline Ingeo biopolymer PLA using s-SSI process showed that loading of thymol (5%) induced formation of larger pores with thinner cell walls (Fig. 5b) compared to the neat foam (Fig. 5a) after same time of soaking in $scCO_2$ (2 h). This could be due to the plasticizing effect of thymol on PLA [28] which led to reduced melt viscosity allowing pores to grow larger during the expansion [32]. Besides uniform and interconnected pores, some bigger pores with a porous inner surface were also observed in the PLA foams obtained *via* d-SSI and SFE-SSI processes in the HPEA unit (Fig. 5c and 5d). This could be due to a premature phase separation resulting from a lower pressure drop and non-uniform depressurization during venting out a higher CO_2 volume, whereby the phase-separated CO_2 fraction makes gas pockets and lead to formation of large cells [33].

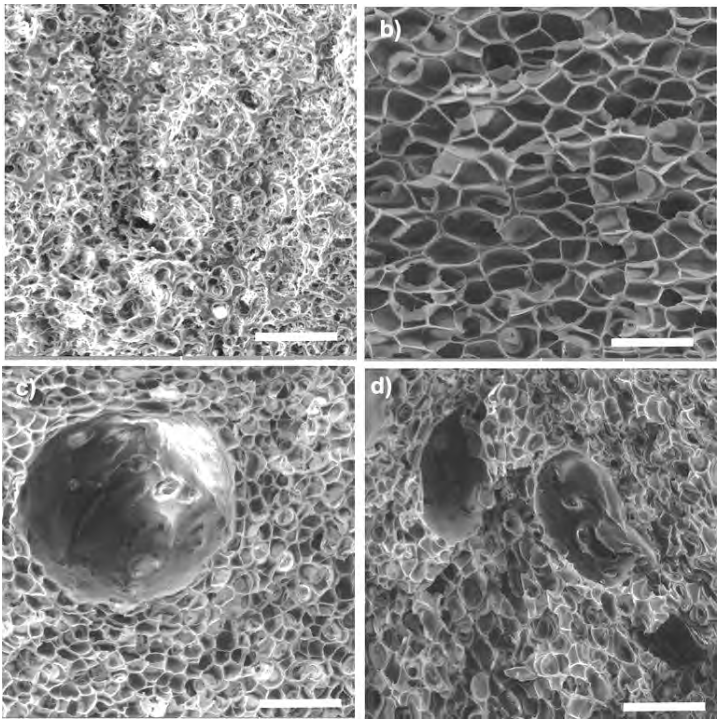

Fig. 5. Morphology of (a) neat PLA foam and foams impregnated with thymol at 30 MPa and 100°C using (b) s-SSI and (c) d-SSI, or (d) with thyme extract using SFE-SSI (scale bar = 100 μm)

Morphology of the PLA film treated with pure $scCO_2$ and soaked in supercritical solution of thymol at 10 MPa and 40°C is presented in Fig. 6. Sufficiently high thymol loading (6.6%) for proper antibacterial effect [3] was achieved upon 2 h of impregnation. This amount of thymol induced negligible swelling extent of the film (1.20%) and had no adverse effect on the compactness or elasticity of film which is important for a potential use in food packaging.

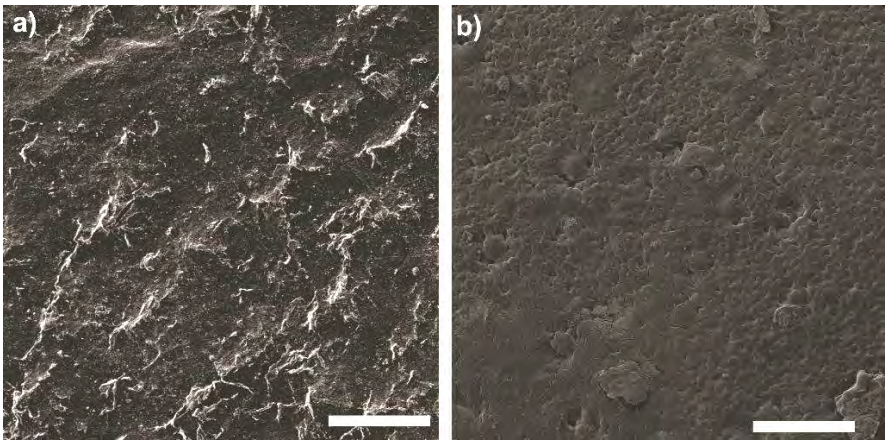

Fig. 6. Morphology of the PLA film (a) neat and (b) impregnated with thymol

The results obtained indicated feasibility of uniting two processes – foaming of PLA and PLGA polymers using $scCO_2$ and their impregnation with active substance. As the result, highly porous material with antibacterial properties was obtained.

2.4. Impregnation of polypropylene non-woven material

Today polypropilene non-woven materials are widely used as medical textiles like disposable surgical gowns, shoe covers, head covers, facemasks, drapes, etc [34]. It is estimated that around 10% of all hospital patients are being infected by hospital acquired infections (HAI) during their hospitalization [35-37]. In that sense, it is of ultimate interest to use medicinal textiles with antibacterial properties.

In order to investigate potential of hospital textile with antibacterial activity fabrication using SSI process a study on polypropylene non-woven material impregnation with thymol in $scCO_2$ was performed [37]. Although easy to impregnate using $scCO_2$, non polar polypropylene fabrics have a significant drawback – long wetting time. In order to eliminate this shortcoming, polypropylene non-woven material was treated with corona discharge at atmospheric pressure with the aim to induce formation of polar groups at the surface of polypropylene fibers. SSI of both (untreated and corona treated polypropylene) with thymol was investigated. Based on preliminary tests and previous investigations, temperature of 35°C, pressure of 15.5 MPa and impregnation time of 4 h were selected to be the SSI parameters, whereby thymol to polypropylene ratio was varied. The results showed no difference in the impregnation yields obtained for untreated and corona treated polypropylene. Depending on the thymol to polypropylene ratio, yields from 0.7% – 11% were obtained. However, there was a significant difference in wetting time between investigated samples. Wetting time of both polypropylene non-woven material

and polypropylene non-woven material impregnated with thymol (impregnation yield of 7.41%) exceeded 20 min whereas water droplet wetted the corona treated polypropylene non-woven material and corona treated polypropylene non-woven material impregnated with thymol (impregnation yield of 6.69%) immediately confirming that corona pre-treatment remarkably improved wettability of the material [37]. The thymol impregnation yield around 7% for both untreated and corona pre-treated polypropylene non-woven fabrics provided maximum microbial reduction (99.9%) against tested *E. coli*, *S. aureus* and *Candida albicans*.

The obtained results indicated that the corona modified modified polyporpylene material with good wettability and strong antibacterial activity could be fabricated using SSI process starting from the corona pretreated material.

2.5. Impregnation of selected starch films

In this part, a few results of the SSI of selected starch based films produced in New Chemical Syntheses Institute (Pulawy, Poland) with thymol will be presented in order to illustrate potential of the SSI process for impregnation of polymer films. The films selected were: corn starch film (CS), thermoplastic potato starch + polyester film (TPS + p) and thermoplastic potato starch + corn starch film (TPS + CS). The SSI was performed at temperature of 35 ^{o}C and pressures of 15 MPa and 30 MPa whereby the impregnation time was varied from 30 min to 5 h. High impregnation yields up to 37% were obtained. Some of the representative results obtained are presented in Table 2.

Table 2. Representative results of the SSI of selected starch based films with thymol

Substrate	P(MPa)/T (°C) /t (h)	Impregnation yield (%)
CS	15/35/2	31.9
TPS + p	30/35/2	16.2
TPS + CS	15/35/2	14.7

2.6. Impregnation of polymers with plant extracts

Due to the wide spectrum of their biological activity, there is a need for impregnation of plant extracts into polymer materials which will enable fabrication of added value materials (e.g. materials with controlled release). This need is especially pronounced when it comes to the plant extracts with strong antibacterial activity because bacterial strains can not develop mechanism of resistance against such mixtures of natural active components.

In case of supercritical extracts impregnation it is the most efficient to combine supercritical extraction and impregnation processes. The combined SFE-SSI process was developed and investigated on the large number of raw materials and polymers within the joint research between Institute of Materials

Science and Technology (INTEMA) (Argentina), Eurotechnica GmbH (Germany) and our team from the University of Belgrade Faculty of Technology and Metallurgy (Serbia) [10, 38, 39]. In this process a stream of supercritical fluid with the dissolved natural components is led from the extractor vessel into the adsorption column where the polymer is placed, which enables energy savings and minimization of losses compared to processes conducted separately. Different modes of processing are possible [10]. Because the adsorption is much slower process than the extraction, circulation of the supercritical solution through the both vessels or through the adsorption vessel is needed [10, 39]. To design the combined process, previous optimization of the SFE (pressure and temperature conditions, flow rate) and the SSI (pressure and temperature conditions, rate of decompression) as well as investigation of the polymer behavior under the high pressure are mandatory. Then, optimization of the combined process (mode of operation as well as the mass ratio plant material/polymer/consumed $scCO_2$) commences. Selected results of the combined process application are presented in Table 3.

Table 3. Selected results related to the combined SFE-SSI process

Natural material	Substrate	Impregnation yield (%)	Literature
Thyme (*Thymus vulgaris*)	Cotton gauze	9.0	[10]
	Cellulose acetate	1.5	
	Polypropylene	4.8	
	Corn starch	0.7	
	Polycaprolactone	9.1	
	Chitosan	1.0	
Lichen (*Usnea lethariiformis*)	Polycaprolactone	2.8	[38]
Hops (*Humulus lupulus*)	Corn starch	2.6	[39]
	Polycaprolactone	6.0	
	Polypropylene	4.4	

3. CONCLUSION

Impregnation process with supercritical carbon dioxide was successfully applied for fabrication of materials with antibacterial properties based on cotton, polypropylene, cellulose acetate, poly(lactic acid), poly(lactic-*co*-glycolic acid), starch, chitosan and polycaprolactone. Carriers of antibacterial activity were thymol, carvacrol or extracts obtained from thyme, hops and lichen (*U. lethariiformis*) via supercritical fluid extraction. Impregnated cotton and polypropylene fibers may have application in medicinal textiles. Potential of cellulose acetate is huge and appropriate to variety of applications due to its high loading capacity for thymol and carvacrol providing release time from few hours to 21 days. Application of SSI to poly(lactic acid) and poly(lactic-*co*-glycolic

acid) enables fabrication of polymer foams with antibacterial properties. Starch based polymer films can be modified by the SSI in order to acquire antibacterial properties for the application in packaging.

Acknowledgments

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SUPERCRITICAL FLUID EXTRACTION OF FENNEL (*Foeniculum vulgare* Mill.) SEED FROM MONTENEGRO: ANTIMICROBIAL ACTIVITY

Summary

Supercritical carbon dioxide (SC-CO_2) extraction of fennel seeds (*Foeniculum vulgare* Mill.) was carried out in this study to assess the effect of the process parameters – mean particle size of fennel seeds and flow rate of CO_2 – on the yield, chemical composition and antimicrobial effect of extracts. The obtained extracts chemical composition was compared to the fennel seed essential oil isolated by hydrodistillation. In SC-CO_2 extracts as well in the hydrodistilled oil the major compounds were *trans*-anethole (61.45–70.09%) and (60.53%), fenchone (7.87–12.68%) and (20.72%), estragole (5.24–7.78%) and (5.01%), respectively.

The study showed that the particle size plays an important role in SC-CO_2 extraction of fennel seeds, and significant increase in extraction rate with decrease of the particle size of fennel seed was noted. Further, results indicate that flow rate of CO_2 does not strongly influence the yield and composition of fennel extracts. The highest activity was observed against *Staphylococcus aureus* and *Escherichia coli* while the lowest activity of SC-CO_2 extracts was observed for *Pseudomonas aeruginosa*.

1. INTRODUCTION

Aromatic plants and herbal extracts have been used for thousands of years as incense, perfumes and cosmetics. After the discovery of plant`s medicinal

properties, natural flora became a valuable source of health improvement in the ancient civilizations.

Considering vascular flora of Montenegro, its richness is estimated on ca 3600 species and sub-species (Stešević and Caković, 2013). If the richness is expressed over the ratios between its surface of area and a number of species, than Montenegro is one of the floristically richest countries in Europe. Data collected from different sources suggest that the number of medicinal plants in Montenegrin flora is about 700, and that 300 of them is wide-used in traditional medicine (Stešević and Jovović, 2008). Aromatic herbs are mainly collected in nature, wild-growing, while cultivation is present only in small family farms.

Fennel (*Foeniculum vulgare* Mill.) is annual, biennial or perennial plant, depending on the variety, belonging to *Apiaceae* family and is native to the Mediterranean area (Barros et al., 2010). It has been cultivated and introduced into many regions outside that zone (Volák and Stodola, 1998). In Montenegro fennel is wild-growing herb, unfortunately not used enough and often considered as weed.

Mature fennel fruit and essential oil are used as flavoring agents in food products such as bread, pastries, liqueurs, pickles, and cheese. They are also used as a constituent in cosmetic and pharmaceutical products (Ruberto et al. 2000; Faudale et al., 2008; Telci et al., 2009). Fennel herbal tea is a well-known household remedy traditionally used for the treatment of a diversity of symptoms of the respiratory and gastrointestinal tract (Raffo et al., 2011). Fennel is also highly recommended for bronchitis and chronic coughs, for diabetes, for the treatment of kidney stones, and has diuretic and stomachic properties (Choi et al. 2004; Novais et al., 2004). Essential oils are traditionally obtained by hydrodistillation or steam distillation. These processes are not expensive but can induce the thermal degradation, hydrolysis and water solubilization of some fragrance constituents (Reverchon, 1997; Della Porta et al, 1998) while extracts obtained by organic solvents might contain residues that pollute the foods and fragrances to which they are added (Damjanović et al., 2003). Supercritical fluid extraction (SFE) extraction compare favorably with above mentioned processes because it allows high yield to be attained while simultaneously shortens process and fragrance that better resembled the flavor profile of the starting spice material (Doneanu et al, 1998; Araus et al., 2009). Moreover, tuning of the process parameters allows improvement of the selectivity of supercritical carbon dioxide (SC-CO_2) towards desirable fractions with complete separation of the phases and obtaining solvent free extract (Ivanović et al., 2010).

As essential oil and extracts from fennel seeds are important ingredients for flavoring pharmaceutical and food industry products, the improvement of the quality of fennel seed oil is of great economic significance. Many researchers investigated fennel oils composition from various origins and have shown that the major components are phenylpropanoid derivatives and monoterpenoids (Oktay et al, 2003; Barazani et al., 2002; Napoli et al., 2010; Renjie et al., 2010; Diaz-Marotto et al. 2006; Ozcan et al., 2006).

In this work, SC-CO_2 extraction of fennel seeds, growing wild in Montenegro, has been investigated. The aim of the work was to find the influence of CO_2 flow rate and fennel seed particle size on the yield, composition and antimicrobial activity of obtained extracts.

2. MATERIALS AND METHODS

2.1. Collection and preparation of herb samples

Many factors can influence the amount of essential oil in aromatic herbs, such as climate and environmental conditions, season of collection; age of plants and, for fennel especially, the stage of ripening of the fruits (Damjanović et al., 2005). To avoid this influence in the present work the ripe, greenish brown seeds of growing wild fennel (*Foeniculum vulgare* Miller) were collected manually from the same collection site in the Podgorica region (central part of Montenegro), within 4 day. A voucher specimen was deposited in Herbarium, Department of Biology, Faculty of Natural Sciences and Mathematics, University of Montenegro. The seeds were air-dried and stored in double layer paper bags at ambient temperature protected from direct light until further analysis.

The particle size distribution was determined by using sieve set Erweka (Germany) of 3150, 2000, 800, 315, 160 and 100 μm. The mill was operated after freezing the herb material with liquid N_2 to avoid the loss and thermal degradation of the volatile compounds. After being separated by a series of sieves, the fractions were kept in an airtight resalable polypropylene bag at 4°C.

In the case of the particle size effect on the extraction rate, yield and extract composition, three different mean particle diameter size were examined: d_o = 1.48 mm (*in toto*); d_1 = 1.28 mm and d_2 = 0.93 mm.

Hydrodistillation procedure

80 g of herb material (d = 1,28 mm) was submitted to hydrodistillation in a Clevenger-type apparatus for 2 hours according to Yugoslav Pharmacopoeia IV. The obtained essential oil was dried over anhydrous sodium sulphate, measured, poured in hermetically sealed dark-glass containers and stored at 4°C until analyzed by GC-MS.

Reagents

Commercial carbon dioxide (99.5% purity, Tehno-gas, Novi Sad, Serbia) was used for extractions. HPLC grade chloroform and methanol were purchased from Riedel-de Haën, Germany. Standard samples for GC-MS analyses were purchased from Fluka, Great Britain.

Supercritical carbon-dioxide (SC-CO_2) extraction

SC-CO_2 extraction was carried out with a laboratory scale high-pressure extraction plant (NOVA – Swiss, Effretikon, Switzerland) described previously

(Pekić et al., 1995). The main part and characteristics (manufacturer specification) of the plant were as follows: the diaphragm-type compressor (up to 1000 bar), extractor with an internal volume of 200 ml (p_{max} = 700 bar), separator with internal volume of 200 ml (p_{max} = 250 bar) and a maximum CO_2 mass flow rate of approximately 5.4 kg/h. For all experiments, the amount of fennel seed sample in the extractor was 80 g and the total extraction time was 4 hours (samples were taken every half an hour). Extractor conditions were 90 bar and 40°C while separator conditions were 14 bar and 25°C. The obtained extracts were measured and stored at 4°C until analyzed by GC/MS.

The influence of the CO_2 flow rate (0.3 kg CO_2/h; 0.5 kg CO_2/h and 0.9 kg CO_2/h), on fennel extraction rate, yield and extract composition was studied at mean particle diameter size d_2 (1.28 mm).

GC-MS Analysis

GC-MS analyses were carried out using a Shimadzu QP 5050 gas chromatograph-mass spectrometer equipped with a PTE-5 capillary column with the same characteristics as the one used in GC. The column temperature was maintained at 60°C for 2 min. and then programmed to increase as follows: 60–300°C at 4°C/min. The temperature was 260 °C at the injection port and 300°C at the interface. The samples, previously dissolved in chloroform: methanol (3:1), were injected (1 μL) in split mode with split ratio of 1:20 and the flow rate of the carrier gas (helium) 1.5 ml/min; inlet pressure, 16.8 kPa. The MS conditions were: the ionization voltage, 70 eV, scanning interval 0.5 s, detector voltage 1.3 kV and m/z range of 40–500. The components were identified by computer searching (using data base program Class 5000) and comparing their mass spectral data with available standards and those in the WILEY229 and the NIST107 mass spectra libraries.

Microbial strains

In order to evaluate the activity of the essential oil of *F. vulgare*, the following microorganisms were used: as reference strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (Torlak, Belgrade, Serbia); further, the clinically isolated *S. aureus*, *E. coli* and *P. aeruginosa*.

The microorganisms were isolated from clinically treated patients of the Clinical Centre of Montenegro (Podgorica, Montenegro).

Determinations of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) (NCCLS, 2003). All tests were performed in Mueller Hinton broth supplemented with Tween 80 at a final concentration of 0.5% (v/v). Briefly,

serial doubling dilutions of the extracts were prepared in a 96-well microtiter plate ranged from 0.09 to 25.00 mg/ml. To each well 10 μL of resazurin indicator solution (prepared by dissolving a 270-mg tablet in 40 mL of sterile distilled water) and 30 μL of Mueller Hinton broth were added. Finally, 10 μL of bacterial suspension (10^6 CFU/mL) was added to each well to achieve a concentration of 10^4 CFU/mL. Two columns in each plate were used as controls: one column with a broad-spectrum antibiotic as a positive control (amykacine) and one column containing the methanol as negative controls. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37°C for 18–24 h. Color change was then assessed visually. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated and that were the MIC and the MBC for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth. The microorganism growth was indicated by the turbidity. The MBC was defined as the lowest concentration of the extract at which incubated microorganism was completely killed.

Statistical analysis

Extraction experiments were carried out in triplicate. Means and standard deviation (SD) were calculated using Origin Pro 8 (OriginLab, USA). The Duncan′s test was conducted to analyze the difference between various pre-treatments. A $P < 0.05$ was considered as statistically significant.

3. RESULTS AND DISCUSSION

The effect of the process parameters, namely CO_2 flow rate and diameter particle size of fennel seeds on the extraction rate and yield is shown in Fig. 1–2, where the yield of extract (g/100 g of dry herb) is plotted versus extraction time (min).

The data from this experiments (Fig. 1) indicates that there is a positive relationship between the effect of CO_2 flow rate and the mass fraction extracted from each sample after 4 hours extraction (from 3.05 g/100 g of dry herb at 0.3 kg/h to 3.75 g/100 g of dry herb at 0.9 kg CO_2/h). This is due to the increased amount of solvent that was exposed to the herb sample.

However, it was found that at CO_2 at flow rate of 0.5 kg/h, after 4 hours of extraction, extract yield was 4.01 g/100 g of dry herb what is rather comparable to highest flow rate yield and much higher CO_2 consumption. Also, previous research found that CO_2 flow rate cause decrease of essential oil content in isolated extract (Gaspar et al., 2000). Similar results were reported previously, where for the highest flow rate the extraction of the fennel oil was faster but extraction curves overlap, after 3 h of extraction (Coelho et al., 2003).

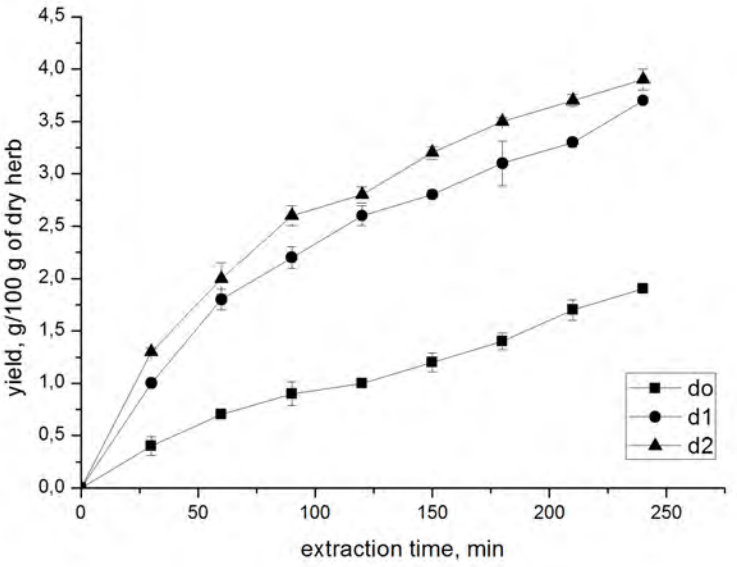

Fig. 1. Yield (g/100 g of dry herb) of fennel seed extracts isolated by SC-CO_2 at different diameter particle size (flow rate 0.5 kg CO_2/h), expressed as mean ± standard deviation

Particle size plays an important role in SC extraction processes, when internal mass transfer resistance is reduced and the extraction is controlled by equilibrium conditions. In our wok, as the size of the seeds reduces, the extraction rate increases (Fig. 2).

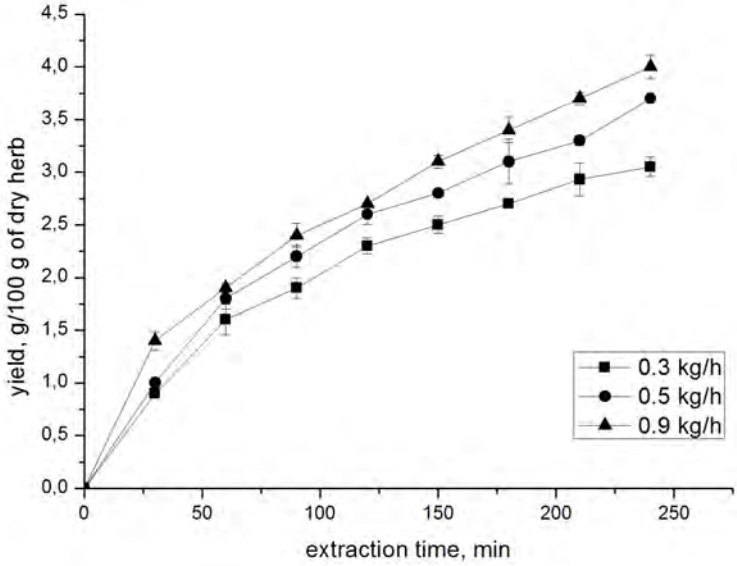

Fig. 2. Yield (g/100 g of dry herb) of fennel seed extracts isolated by SCE at different CO_2 flow rate (d_1 = 1.28 mm), expressed as mean ± standard deviation

Thus, at the highest diameter particle size (intact herb), after 4 h CO_2 extraction, the extraction yield was 1.92 g/100 g of dry herb which was much lower comparing to the yield of 3.72 and 3.94 g/100 g of dry herb (diameter particle size 1.28 and 0.93 mm, respectively). This can be explained by the positive effect of a reduced particle size on the internal resistance to mass transfer in the solid matrix. In fact, the extraction rate increased because of the shortening in the diffusion path. Thus, entrapped essential oils liberate by the disruption of the glands and become easily accessible for extraction. It was previously found (Reis-Vasco et al., 2000; Aleksovski and Sovová, 2007; Fornari et al., 2012) that reduced substrate particle size positively effects essential oils yield and SC-CO_2 extraction rate. Smaller particles caused increases in the specific surface area as well as a disruption of the cell walls and other inner barriers, thus leaving the essential oil more reachable to SC-CO_2. In an intact herb matrix, compressed CO_2 easily reach the wax components from the seeds surface, which is not case with essential oil. Our results differ from previously reported, where extraction curves overlap for different fennel seed particle size as function of the extraction time, at a fixed flow rate (Coelho et al., 2003).

Thirty compounds were identified in extracts obtained after 4 h supercritical CO_2 extraction of fennel seeds (Table 1). Major compounds were *trans*-anethole (61.45–70.09%), fenchone (7.87–12.68%), estragole (5.24–7.78%), tetradecene (1.61–5.24%) and 7-octadecanone (2.44–8.73%). Eighteen compounds were identified in essential oil obtained by hydrodistillation. Major compounds in oil were *trans*-anethole (60.53%), fenchone (20.72%) and estragole (5.01%).

The optimal chemical composition of fennel seed essential oil is depends on the desired oil application. In food industry, the most suitable is fennel oil with high percentage of *trans*-anethole, responsible for fennel seed aroma, while pharmaceutical industry prefers essential oil with higher percentage of fenchone. In both cases, content of estragole, which is assumed to be toxic to a certain degree, should be reduced (Damjanović et al., 2003).

It can be seen from Table 1 that with the CO_2 flow rate incensement, content of *trans*-anethole decrease (from 66.34% to 62.29%), while content of estragole increase (from 5.44% to 7.78%). Estragole has been demonstrated to be genotoxic and carcinogenic and, consequently, reduction in exposure and restrictions in use levels as flavoring substance has been recommended by the European Scientific Committee on Food. Thus, it was established maximum levels for estragole in certain compound foods resulting from the use of food ingredients in which it naturally occurs (Raffo et al., 2011).

On the other hand it can be seen that content of fenchone is nearly doubled in sample obtained at 0.5 kg CO_2/h (13.68%) comparing to extracts obtained at CO_2 flow rate 0.3 and 0.9 kg CO_2/h (8.41% and 7.87%, respectively).

A comparison between the essential oil obtained by hydrodistillation and SC-CO_2 extracts showed that in latter technique some undesired higher molecular weight compounds (waxes) were co-extracted with essential oil (Table 1). The co-extracted waxes consist of paraffin as well as fatty acids,

alcohols, esters, aldehydes and ketons. The major compounds were hexacosane (0.44–1.95%), tetradecene (4.09–5.97%) and 7-octadecanone (3.47–8.73%). The content of nonvolatile compounds was comparable at lower CO_2 flow rate (12.81% and 12.11%) while it was visibly higher at the highest flow rate (19.23%). It was previously stated that the increase of flow rate led to decrease in the essential oil concentration (Gaspar et al., 2000). The content of oxygenated compounds, which strongly contributed to the fragrance, was lowest in sample obtained at the highest flow rate (77.1%). Thus, our work confirms that use of low flow rates is favorable for extraction of most important compounds of fennel seed essential oil.

Table 1. Relative percentage composition of fennel seed SC-CO_2 extracts and hydrodistilled essential oil

Compound	1.28 mm 0.3 kg/h	1.28 mm 0.5 kg/h	1.28 mm 0.9 kg/h	1.49 mm 0.5 kg/h	0.93 mm 0.5 kg/h	HD
α-thujone	tr	tr	tr	tr	tr	tr
α-pinene	0.22	0.48	0.13	tr	0.24	2.79
camphene	-	0.02	-	-	0.07	3.01
sabinene	0.1	0.12	0.07	-	0.17	0.58
β-pinene	tr	tr	tr	tr	tr	tr
myrcene	0.63	0.76	0.62	tr	0.39	2.62
α-phelandrene	0.07	0.31	0.14	tr	0.15	0.68
p-cymene	tr	0.20	tr	-	0.05	0.32
limonene	0.86	1.29	0.37	0.25	0.55	3.28
1,8 cineole	0.39	0.52	0.28	0.19	0.20	1.12
β-ocimene	tr	tr	tr	tr	-	0.18
γ-terpinene	0.85	0.63	0.87	0.29	0.37	1.17
fenchone	8.41	12.68	7.87	8.37	11.21	20.72
camphor	0.50	0.12	tr	tr	tr	0.52
terpinene-4-ol	-	-	-	-	0.09	-
estragole	5.44	6.67	7.78	5.71	5.24	5.01
cis-anethole	tr	tr	tr	tr	tr	tr
anisaldehyde	0.15			-	0.16	-
trans-anethole	66.34	62.29	61.45	61.78	70.09	60.53
germacrene D	0.76	0.34	0.27	0.23	0.25	0.89
tetradecanoic acid	0.23	0.23	0.96	0.67	0.13	-
hexadecanoic acid	0.09	0.32	0.98	0.78	0.65	-
tetradecane	5.95	4.12	5.24	4.97	1.61	-
1,2 benzenedicarboxylic acid, dioctyl ester	0.80	0.62	0.56	0.61	0.34	-
pentadecane	0.54	0.24	0.55	1.02	0.07	-
pentacosane	0.76	0.28	0.88	0.97	0.19	-
1-hexadecanol	0.77	0.82	1.21	1.27	0.34	-
hexacosane	0.44	1.11	1.88	1.95	1.21	-
1-octadecanol	0.79	1.22	1.49	1.11	1.38	-
7-octadecanone	2.44	3.15	5.48	8.73	3.47	-
Total identified	97.53	98.54	99.08	98.90	98.62	98.72

It can be seen from Fig 3. that the content of monoterpene hydrocarbons was noticeably higher in sample obtained at 0.5 kgCO_2/h (4.33%) comparing to samples obtained at 0.3 a nd 0.9 kgCO_2/h (2.62% and 2.48%, respectively). This is namely due to incensement of limonene, which have been shown to possess antiviral activity, helping liver detoxication and secretion of cancerogenic compounds (Crowell et al., 1994).

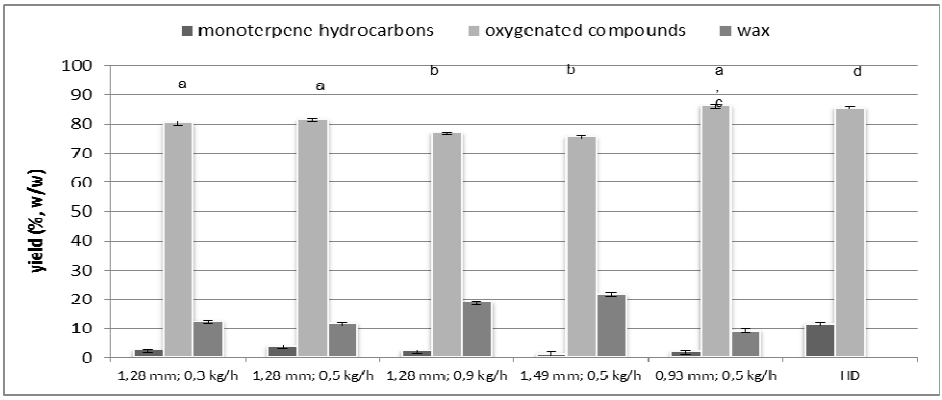

Fig. 3. Yield (%) of fennel seed extracts isolated by SC-CO_2 extraction and hydrodistillation (HD) with respect to grouped components, expressed as mean ± standard deviation; [a,b,c,d]Means for each pre-tretmeant, without a common letter, are significantly different from each other ($P < 0.05$)

Content of non-volatile compounds is the highest in the intact herb sample (22.08%) while it decrease as the size of the seeds reduces, for particle size 1.28 mm and 0.93 mm (19.23% and 9.39%, respectively). It is obvious that as the size of the seeds reduces, the content of oxygenated compounds increase, from a minimum of 75.86% at 1.49 mm, over 77.1% at 1.28 mm to a maximum of 86.69% at 0.93 mm. It is mainly because of the increasement of content of trans-anethole, which is present in percentage of 70.09% in 0.93 mm, while 61.78% and 61.45% in 1.49 mm and 1.28 mm, respectively. Therapeutically, trans-anethole is an important compound that has been shown to possess anti-inflammatory and antifungal activities (De at al., 2002).

The results of the bioassays (Table 2), show that tested *F. vulgare* extracts obtained by SC-CO_2 extraction weaker or similar antimicrobial activity in comparison with essential oil obtained by hydrodistillation.

The highest antibacterial efficiency showed SC-CO_2 extract obtained from the sample with lowest diameter particle size, where MIC ranged between 0.09 mg/ml and 25.0 mg/ml. The highest activity was observed against *S. aureus* and *E. coli* (0.09 mg/ml), what confirms previous results on this subject (Mohsenzadeh et al., 2007). This efficiency could be attributed to the high content of compounds with known antimicrobial activity, such as phenylpropanoid *trans*-anethole and its isomers (Diao et al., 2014; Patra at al.,

2002). The lowest activity of SC-CO_2 extracts was observed for *P. aeruginosa* (25.0 mg/ml). However, Saviuc et al. (2012) reported antibacterial potential of fennel oil against *P. aeruginosa* which was not observed in the present investigation.

Table 2. Antimicrobial activity of *F. vulgare* extracts obtained by SC-CO_2 extraction (after 4h) and essential oil obtained by hydrodistillation

Process parameters	1.28 mm 0.3 kg/h	1.28 mm 0.5 kg/h	1.28 mm 0.9 kg/h	1.49 mm 0.5 kg/h	0.93 mm 0.5 kg/h	HD
Microorganisms	MIC/MBC (mg/ml)					
Staphylococcus aureus	0.36/0.72	1.57/3.13	0.72/0.72	1.57/1.57	0.36/0.36	0.18/0.36
S. aureus ATCC 25923	0.18/0.36	0.72/0.72	0.36/0.36	0.72/1.57	0.09/0.18	0.18/0.18
Escherichia coli	3.13/6.25	0.36/0.72	0.72/0.72	3.13/6.25	0.36/0.36	0.18/0.36
E. coli ATCC 25922	0.18/0.36	0.36/0.72	0.36/0.72	0.36/0.72	0.09/0.18	0.09/0.18
Pseudomonas aeruginosa	>25.0	>25.0	>25.0	>25.0	12.5/25.0	25.0/25.0
P. aeruginosa ATCC 27853	6.25/12.5	12.5/25.0	12.5/12.5	12.5/12.5	6.25/12.5	6.25/6.25

The weakest antimicrobial efficiency showed SC-CO_2 extract obtained from the sample with highest diameter particle size (MIC ranged between 0.36 mg/ml and 25.0 mg/ml) probably due to the smallest content of very important oxygenated compounds.

The mentioned results suggest the significance of individual oil components percentage ratio in the antimicrobial mixture. Antimicrobial action is often determined by more than one component; each of them contributes to the beneficial or adverse effects (Araus et al., 2009). Antimicrobial activity of obtained fennel extracts decrease as CO_2 flow rate incease, probably due to content of *trans*-anethole and fenchone.

The obtained results showed that SC-CO_2 extracts as well as essential oil obtained by hydrodistillation were more effective against ATCC strains than against clinically isolated strains. The data indicated that Gram-positive *E. coli* was the most sensitive strain tested to the savory extracts while *P. aeruginosa* was the most resistent. Gram-negative *P. aeruginosa* is recognized to have a high level of intrinsic resistance to almost all known antimicrobials and antibiotics, due to a very restrictive outer membrane barrier (Mann et al., 2000). *Pseudomonas* species are used extensively in bioremediation as known to have the ability to metabolise a wide range of organic compounds. Thus, they may simply metabolise the compounds in extracts that are inhibitory to many of the other bacteria (Serrano et al., 2011).

4. CONCLUSIONS

In this study, extracts were obtained from fennel (*Foeniculum vulgare* Mill.) by tuning CO_2 flow rate and seeds in order to obtain extract with best characteristics. It was found that, the extraction yield was significantly enhanced as particle diameter size decrease while increasing flow rate just slightly positive influence extraction yield of fennel seed extracts. Content of very imoprtant compound *trans*-anethole decrease with the CO_2 flow rate incensement, while content of estragole increase (from 5.44% to 7.78%). The content of oxygenated compounds, which strongly contributed to the fragrance, increase as the size of the seeds reduces and was highest in sample obtained at the lowest flow rate. The highest antibacterial efficiency showed SC-CO_2 extract obtained from the sample with lowest diameter particle size, where MIC ranged between 0.09 mg/ml and 25.0 mg/ml while antimicrobial activity decrease as CO_2 flow rate increase, probably due to content of *trans*-anethole and fenchone.

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POSSIBILITIES AND ADVANTAGES IN THE APPLICATION OF SUPERCRITICAL EXTRACTS AS ACTIVE INGREDIENTS IN COSMETIC PRODUCTS

Summary

Today, there is a growing consumer demand for cosmetic products containing natural and/or organic ingredients. These natural ingredients used in cosmetic products include a variety of preparations, such as plant extracts, expressed juices, tinctures, waxes, vegetable oils, lipids, plant carbohydrates, essential oils, as well as purified plant components, such as vitamins, antioxidants or other substances with biological activity. Herbal extracts are primarily added to the cosmetic formulations due to several associated properties such as antioxidant, anti-inflammatory, antiseptic and antimicrobial activities. Due to increasingly stringent environmental regulations, supercritical fluid extraction (SFE) has gained wide acceptance in recent years as an alternative to conventional solvent extraction for separation of organic compounds in many analytical and industrial processes. In the past decade, SFE has been applied successfully to the extraction of a variety of organic compounds from plant materials with a lot of advantages in comparison to conventional extracting methods. This review article presents the practical aspects of application of supercritical herbal extracts as ingredients in cosmetic products as well as advantages of SFE as novel method for liposomal microencapsulation.

1. INTRODUCTION

It is probably safe to say that plant derived ingredients were among the very first cosmetics. Natural colorants, plant juices for soothing and protection

from insect pests, and fragrant oils for imparting odor were all known and used in ancient times. Although there has always been a continuing interest in the use of ingredients derived from plants in cosmetics, in the beginning of the 1990s, that interest exploded, with new discoveries of benefits, greater standardization and control of raw material specifications, and new formulation techniques [1].

Botanical ingredients used in cosmetic products include a variety of preparations, such as plant extracts, expressed juices, tinctures, waxes, vegetable oils, lipids, plant carbohydrates, essential oils, as well as purified plant components, such as vitamins, antioxidants or other substances with biological activity. The variety of plants providing these ingredients ranges from staple food plants (cereals, fruits, vegetables, roots, bulbs, spices) to herbs used in traditional medicines or teas as well as exotic plants and their ingredients [2–5].

Herbal extracts are primarily added to the cosmetic formulations due to several associated properties such as antioxidant, anti-inflammatory, antiseptic and antimicrobial properties [6–8]. The traditional extraction methods used to obtain these extracts have several drawbacks; thus, they are time consuming, laborious, have low selectivity and/or low extraction yields. Moreover, these techniques employ large amounts of toxic solvents. At present, new extraction methods able to overcome the above mentioned drawbacks are being studied, among them, Supercritical Fluid Extraction (SFE) is among the more promising processes. This extraction technique provides higher selectivity, shorter extraction time and do not use toxic organic solvents [9–11].

2. SUPERCRITICAL FLUID EXTRACTION (SFE)

When a certain fluid is forced to a pressure and temperature higher than its critical point, it becomes a supercritical fluid. Under these conditions, the different properties of the fluid are placed between those of a gas and those of a liquid. Although a supercritical fluid density is similar to a liquid and its viscosity is similar to a gas, its diffusivity is intermediate. Thus, the supercritical state of a fluid has been defined as a state in which liquid and gas are indistinguishable to each other, or as a state in which the fluid is compressible even though posses a density similar of a liquid and, therefore, has its solvating power [12].

Because of its different physico-chemical properties, SFE provides several operational advantages over traditional extraction methods [13]. Due to their low viscosity and relatively high diffusivity, supercritical fluids have better transport properties than liquids, can diffuse easily through solid materials and therefore allow to obtain higher extraction yields. One of the main characteristics of a supercritical fluid is the possibility to modify the density of the fluid by changing the pressure and/or the temperature [9]. Since density is directly

related to solubility by tuning the extraction pressure, the solvent strength of the fluid can be modified changing in this way the selectivity of the system [14]. Other advantages, compared to other extraction techniques, are the use of solvents generally recognized as safe (GRAS), the efficiency of the extraction process (when working with dynamic extraction in which fresh supercritical fluid is forced to pass through the sample), and the possibility of direct coupling with analytical chromatographic techniques such as Gas Chromatography (GC) or Supercritical Fluid Chromatography (SFC) [9].

As for the solvents, there is a wide range of compounds that can be used as supercritical fluids, being carbon dioxide the most commonly used because of its moderate critical temperature (31.3ºC) and pressure (72.8 bar). Besides, supercritical CO_2 gives additional advantages to those inherent to the technique. Carbon dioxide is a gas at room temperature, so once the extraction is completed, and the system decompressed, a complete elimination of CO_2 is achieved without residues and the extract obtained remains free of solvent. At industrial scale, when carbon dioxide consumption is high, the operation can be controlled to recycle it [9].

From the viewpoint of pharmaceutical, cosmeceutical and food applications supercritical CO_2 it is a good solvent, because it is non-toxic, non-flammable, inexpensive, easy to remove from the product and its critical temperature and pressure are relatively low. These properties make it important for natural products sample preparation, is the ability of SFE using CO_2 to be operated at low temperatures using a non-oxidant medium, which allows the extraction of thermally labile or easily oxidized compounds. It is environmental friendly and generally recognized as safe by FDA and EFSA [15].

Supercritical CO_2 is suitable for extracting, for example, thermally labile and non-polar bioactive compounds but, because of its non-polar nature, it cannot be used for dissolving polar molecules. The solubility of polar compounds and the selectivity of the process can be increased by adding small quantities of other solvents, such as ethanol, in the fluid as co-solvent or modifier. On one hand, it decreases the processing times, increases yields and makes it possible to use milder processing conditions, but on the other, it complicates system thermodynamics and increases capital costs [15]. The other drawbacks are its high initial investment and difficulties to perform continuous extractions [16].

3. SUPERCRITICAL FLUID EXTRACTION OF TOCOPHEROLS

In nature, vitamin E consists of four tocopherols (α-, β-, γ-, and δ- tocopherol) and four tocotrienols (α-, β-, γ-, and δ-tocotrienols), determined by the numbers and position of methyl groups (−CH3) present on the chromanol ring. The tocopherols and tocotrienols are collectively referred to as tocochromanols or tocols. Vitamin E is the major bioactive constituent of human

diet and is well-known for its potent antioxidant and anticancer activities. It is also receiving growing attention in cosmetic and clinical dermatology because of its photoprotection and antioxidant properties [16]. These are the reasons for an increasing interest of vitamin E extraction from natural sources. Besides its well-known antioxidant activity, recent studies have demonstrated synthetic vitamin E to be less effective than natural vitamin E [17].

SFE offers several advantages for the enrichment of tocochromanols over conventional techniques such as vacuum distillation, in particular a lower operating temperature. Tocochromanols exhibit an intermediate solubility between the oil and water and good solubility in liquid CO_2. This property is used for extraction and purification of these compounds is possible, e.g. with adsorptive or chromatographic techniques, again using supercritical fluids to obtain pure compound [15].

Several natural sources have been used to isolate vitamin E using supercritical carbon dioxide extraction. Hadolin et al. (2001) studied the extraction of vitamin E-rich oil from a plant (*Silybum marianum*) that naturally grows in Mediterranean area. It was pointed out that extractions at 60ºC and 200 bar produced the most concentrated extracts in terms of α-tocopherol (0.08%), while the extraction yield was relatively high (19%) [18]. Other important source of vitamin E is the wheat germ. Ge et al. (2002) extracted vitamin E from this material at the following extracting conditions: 275 bar, 40ºC and a CO_2 flow rate of 2 ml/min for 90 minutes. The amount of total vitamin E extracted at these conditions was higher than those obtained using traditional extraction methods (with n-hexane or chloroform/methanol mixtures). Likewise, the quantities of α, γ and δ-tocopherol were much higher using SFE. However, the n-hexane extracts, and mainly, the chloroform/methanol extracts, showed higher selectivity to β-tocopherol [19].

Imsanguan et al. (2008) comparatively studied the efficiency of supercritical CO_2, solvent and soxhlet extraction methods to extract α-tocopherol from rice bran, and found the highest extractability of α-tocopherol with supercritical CO_2 extraction. In supercritical CO_2 extraction method, 48 MPa pressure, and 55°C temperature was found optimum for maximum extractability of α-tocopherol from rice bran. The author also observed that neither ethanol nor hexane can extract α-tocopherol at atmospheric pressure, however, under soxhlet extraction, hexane was found better than ethanol [20].

4. SUPERCRITICAL FLUID EXTRACTION OF GRAPE POMACE

Grape (*Vitis vinifera* L.) is one of the major crop produced worldwide. Its utilizations include fruit consumption, pharmaceutics and wine making (from 70 to 80%). Residue of wine making is named grape pomace and accounts for 20% of grape (w/w). It is composed of seeds, 38 to 52% on a dry matter basis, but also of stems, pulps and skins. The presence of oil and the high phenolic

content of grape seeds offers alternative valorization pathways for these by-products [21].

The total extractable phenolics in grapes are present at about 10% in the pulp, 60%–70% in the seeds and 28%–35% in the grape skin [22]. *Vitis vinifera* grape seed extract is reported to function as anti-caries agent, antidandruff, antifungal, antimicrobial, antioxidant, flavoring, light stabilizer and sunscreen agent [23].

Polyphenolic proanthocyanidins, which in turn can bind to each other to form oligomers known as procyanidins are strong antioxidants compared with vitamin C and E. By inhibition of lipid peroxidation they facilitate wound healing and protect collagen and elastin from degradation. Grape seed extracts show tyrosinase-inhibiting activity, and are useful in antiaging and skin-lightening cosmetics. It was also discovered that proanthocyanidins extracted from grape seeds promote proliferation of hair follicle cells in vitro and that they possess remarkable haircycle-converting activity from the telogen phase to the anagen phase in vivo [24]. Proanthocyanidins potentially improve chloasma in a short period of administration. Yamakoshi et al. reported the oral administration of a proanthocyanidin rich extract from grape seeds for one year reduced effectively the hyperpigmentation of women with chloasma [25].

Grape seed extract exerts a powerful antioxidant effect to bond with collagen, and has been shown to notably protect the body from premature (skin) aging. Also, grape wine extracts exert protective effects against aging in general, notably because of the presence of the antioxidant polyphenol resveratrol. Thereby, in an in vitro study using a three-dimensional tissue culture model of human epidermis, a grape wine extract was able to prevent skin oxidative damage induced by UVB exposure (e.g., reduction of GSH and inflammatory molecules such as IL-1 and PGE) [26].

Oliviera et al. (2013) evaluated the global extraction yield, the antimicrobial activity and the composition profile of Merlot and Syrah grape pomace extracts obtained by supercritical CO_2 and CO_2 added with co- solvent at pressures up to 300 bar and temperatures of 50 and 60°C. The results were compared with the ones obtained by Soxhlet and by ultrasound-assisted leaching extraction methods. The main components from the extracts, identified by HPLC, were gallic acid, p-OH-benzoic acid, vanillic acid and epicatechin. The antibacterial and antifungal activities of the extracts were evaluated using four strains of bacteria (Staphylococcus aureus, Bacillus cereus, Escherichia coli and Pseudomonas aeruginosa) and three fungi strains (Candida albicans, Candida parapsilosis, Candida krusei). Despite lower extraction yield results, the supercritical fluid extracts presented the highest antimicrobial effectiveness compared to the other grape pomace extracts due to the presence of antimicrobial active compounds. Syrah extracts were less efficient against the microorganisms tested and Merlot extracts were more active against Gram-positive bacteria [27].

To produce a high quality extract for use in food, cosmetic and pharmaceutical industry, alternative SFE methods to extract polyphenols from

grape pomace were developed. Due to the polarity of polyphenols, the addition of a modifier to supercritical CO_2 (SC-CO_2) is needed. Da Porto et al. (2014) concluded that ethanol at 15% did not demonstrate appropriate extraction efficiency for polyphenols. Conversely, purified water at 15% proved to be a good modifier. Two alternatives were tested: SC-CO_2 plus water, and SC-CO_2 plus water followed by SC-CO_2 plus ethanol. This second procedure provided the best results favoring the extraction of proanthocyanidins, the major contributors of the antioxidant activity of grape pomace [28].

5. SUPERCRITICAL FLUID EXTRACTION OF TURMERIC

Turmeric (*Curcuma longa*, L.) is best known as a spice used primarily in Asian cuisine, particularly curry, and in prepared mustard. It is also used in some traditional Indian communities as a topical burn treatment. Curcumin (diferuloylmethane), the key biologically active component of turmeric, has shown great potency against acute inflammation and has been shown to exhibit significant wound healing and antioxidant properties. The paste of turmeric powder has been used as antiseptic and for skin nourishment since centuries. Curcumin is a polyphenol used in skin care preparations for treatment or control of psoriasis and other skin conditions such as acne, wounds, burns, eczema, sun damage to the skin and premature aging, due to inhibiting the activity of phosphorylase kinase [24, 29].

In order to obtain curcumin concentrated extracts Martinez-Correaa et al. (2016) employed one-step and two-step extraction models. The one step process involves supercritical carbon dioxide at 60°C and 400 bar, ethanol at 25°C and water at 60°C, both at atmospheric pressure. Ethanol and/or water were used as solvents. In the two-step process, sequential extraction, using supercritical CO_2, was performed followed by ethanol or water extraction. All the extracts obtained were analyzed and results were reported for global extraction yield, the total phenolic content, total flavonoids, curcumin, antimalarial activity and antioxidant activity. The two-step process showed the highest global yield (23.4%) when water is used as solvent in the second step. However, high concentrations of curcumin and high antioxidant activity were obtained when ethanol is used as solvent. The supercritical extract presents low concentration of curcumin and high antimalarial activity; which shows that extracts of different compositions and functionality can be obtained from a sequential extraction [30].

6. SUPERCRITICAL FLUID EXTRACTION OF FENUGREEK SEED

Fenugreek (*Trigonella foenum-graeceum* L.) is a fragrant herb whose seeds have been used medicinally all through the ages, for medicinal and culinary purposes. The seeds of fenugreek are primarily composed of galactomannans.

Galactomannan is a natural effective ingredient for improving skin hydration, possibly through a humectant mechanism [31].

The seeds possess astringent, cooling, demulcent and emollient properties. It offers many dermatological solutions for complete skin and mucous membrane. Cosmetic applications are in hair care, hair loss, hair growth, skin cleansing, skin toning and stimulation. Externally, the seed extracts are emollient and accelerate healing of suppurations and inflammations. Also, many results of the antioxidative properties of fenugreek seed have been reported [24, 30].

Nearly 45% of fenugreek seeds are comprised of the polysaccharide galactomannan. There are developed processes to source the oligo-galactomannanes from fenugreek. The resulting active is said to prevent glycation on collagen and integrin to maintain cutaneous flexibility and elasticity. Also, the active reportedly reduces keratinocyte differentiation of the epidermis to improve cutaneous barriers. As a result of the above two actions, the structure of the skin is preserved and elasticity and firmness are enhanced, improving the appearance of wrinkles [32].

Bogdanovic et al. (2016) analyzed supercritical CO_2 (SC-CO_2) extraction of fenugreek seeds using different pressure and temperature (16.6–33.4 MPa and, 30–50°C), which corresponded to SC-CO_2 density between 779 and 957 kg/m^3. The yield of extract as well as its composition were determined and analyzed for different solvent consumption defined as mass of used CO_2 for extraction per mass of dry fenugreek seeds. A central composite design (CCD) combined with response surface methodology (RSM) was used to study the best extraction conditions which determined that 27.0 MPa, 42.6°C, and 24.6 g CO_2/gdm were the optimal conditions for isolation of total extract. The calculated yield of extract at these conditions defined by SC-CO_2 density of 890 kg/m^3 could be 2.57% based on fenugreek seed. Moreover, the best extraction conditions for obtaining extract which contains the maximal amount of sterols, or maximal amount of vitamin E or vitamin D were also analyzed. These RSM optimizations showed that at slightly lower pressure (22.8–24.8 MPa), temperature (39.9–42.0°C) and consumption of SC-CO_2 (19.0–19.7 g CO_2/gdm) is followed by smaller amount of isolated extract but with maximum content of sterols (16.28%), or vitamin E or vitamin D (1.44% and 0.71%, respectively) at these conditions [33].

7. SUPERCRITICAL FLUID EXTRACTION OF LAVENDER

Essential oils distilled from members of the genus Lavandula have been used both cosmetically and therapeutically for centuries. It is extensively employed in all types of soaps, lotions and perfumes, with the most commonly used species being *Lavandula angustifolia*, *L. latifolia*, *L. stoechas* and *L. x intermedia*. Among the claims made for lavender oil are that it is antibacterial, antifungal and effective for burns and insect bites. This oil in the herbal tradition is said to encourage cell growth and so should be used to help with mending and regeneration in all kinds of skin ailments: bites, stings, boils, burns, stretch

marks, rashes, spots, cold sores, sunburns. Lavender oil inhibits immediate-type allergic reaction in mice and rats. Topical and intradermal lavender oil inhibited the ear swelling response in mice and passive cutaneous anaphylaxis in rats. Peritoneal mast cells were also inhibited from releasing histamine or tumour necrosis factor in vitro when lavender oil was applied [24].

Costa et al. (2012) compared the chemical profiles of bioactive essential oil and extracts obtained by hydrodistillation (HD) and supercritical fluid extraction from *Lavandula viridis*. The SFE was performed at 40°C and at extraction pressures of 12 or 18 MPa in two different separators. They found that SFE achieves the highest yield of bioactive compounds from *L. viridis* but that an increase in the extraction pressure does not produce any further benefits. The chemical analysis of *L. viridis* essential oil and SFE extracts highlighted the abundance of oxygen-containing monoterpenes but revealed significant differences in the chemical profiles. More compounds were identified following the extraction of *L. viridis* essential oil by HD than by SFE. The essential oil and first separator SFE extracts possessed remarkable antioxidant activity [34].

8. SUPERCRITICAL FLUID EXTRACTION OF THYME

Several members of the genus Thymus are cultivated commercially like *Thymus vulgaris* and *Thymus zygis* for their essential oil which is rich in phenolic monoterpenes such as thymol and carvacrol. Both thymol and carvacrol are listed by the United States Food and Drug Administration (FDA) as food additives and currently have the GRAS status. Thyme is commonly used as a culinary herb, and thyme oil and extract are used for food flavoring, manufacture of perfumes and cosmetics, but also for medicinal purposes. Internally, thyme is used for acute bronchitis, laryngitis, whooping cough, chronic gastritis, diarrhea, and lack of appetite. It is also used externally in baths to help rheumatic and skin problems (bruises, sprains, fungal infections) as well as for minor arthritis, gum disease, tonsillitis, etc. [35]. Thyme essential oil is also used in toothpastes, soaps, detergents, creams, lotions, and perfumes. It is claimed to inhibit dandruff, and used in a scalp rub it prevents hair falling out, and rinses containing rosemary and thyme promote natural hair health [24].

Petrovic et al. (2016) investigated the chemical composition, antimicrobial and antioxidant activity of *Thymus praecoxextracts* obtained by fractional supercritical fluid extraction with carbon dioxide. They compared properties of supercritical extracts and extracts obtained by Soxhlet extraction. All investigated extracts showed remarkable antimicrobial activity. Thymol was the major component identified in supercritical extracts which showed the strongest antimicrobial activity. In case of supercritical extracts the fungi appear to be more sensitive compared to the bacteria. Contrary in case of hexane/ethanol extract, the bacteria appear to be more sensitive compared to the fungi [35]. In DPPH assays hexane/ethanol extract of *T. praecox* showed more potent

antioxidant activity than supercritical extracts and synthetic antioxidant BHT. This indicated that the hexane/ethanol extract is rich in phenolics antioxidants. Petrovic et al. (2016) concluded that further studies are necessary to investigate the total phenolic content in extracts. In the case of supercritical extract other conditions i.e. the use of co-solvents, and performing SFE at higher pressure and temperature are necessary to increase content of polyphenols and antioxidant activity, as well as investigation on antioxidant activity by other methods than DPPH assay. The results they presented may suggest that the *T. praecox* extracts are a potential source of antimicrobial ingredients for the food, cosmetic and pharmaceutical industry [35].

The study by Zekovic et al. (2000) reported the SFE of *T. vulgaris* at 40°C and pressures from 80 to 40 MPa. Extracts with the highest thymol content (37.3 wt%) were obtained at 10 MPa and 40°C, with a total extraction yield (mass extracted/thyme load) of 2.1%. Other terpenoids present in the extracts were carvacrol, menthol, camphor and phytol, which were present in quite lower amounts (0.5–7.6 wt%). Additionally, around 40 wt% of hydrocarbons were also identified in their extracts. [36,37].

9. SUPERCRITICAL FLUID EXTRACTION OF CHAMOMILE FLOWER

German chamomile (*Matricaria recutita or Chamomilla recutita*) has been recognized for its therapeutic, soothing properties since the ancient times [24]. Extracts of the plant are used in the form of ointments, lotions and inhalations intended for local application. Chamomile extract, essential oil and isolated constituents, possess anti-inflammatory effects and are useful for treating inflammation of skin and mucous membranes, eczema and as an antipruritic adjunct in the treatment and prevention of skin disorders. Chamomile owes its activity to the presence of flavonoids in the extracts and in the essential oil. Apigenin and its glycosides possess anti-inflammatory, antierythema and antipruritic effects, and so reduce itching and improve the speed at which damaged skin heals by local administration. Alpha bisabolol (levomenol) and chamazulene contribute to the anti-inflammatory activity of chamomile extract by inhibiting leukotriene synthesis and additional antioxidative effects [24]. Chamomile is thought to impart significant cutaneous benefits, such as improving texture and elasticity, as well as reducing pruritus and signs of photo damage, and chemical assays have suggested that chamomile exhibits some antioxidant activity. Chamomile is included in skin formulations as an emollient and to provide anti-inflammatory action for sensitive skin, may help in whitening age spots, take the soreness out of a boil, minor wound, burn, or an insect bite, or is used for dry skin, windburn, sunburn, or even chronic skin conditions such as acne and psoriasis [30, 38].

The chamomile flower heads were extracted in lab scale with CO_2 at pressures 100, 150 and 250 bar and temperatures 30 and 40°C. According to

extraction yield the best extraction conditions were 250 bar and 40°C where the yield was 3.81%. Extraction yield increased with increasing solvent density and at constant solvent density the extraction yield was increased with higher temperature. According to the content of active compounds in extracts, the best extraction conditions one step separation were determined to be 250 bar, 30°C, where the content of matricine reached 9.81 mg/g. The content of matricine in extracts could be even increased by using two step separation procedure and by using this technique the highest content of matricine in extract obtained was 28 mg/g (extraction at 40°C and 250 bar). The results indicate that lab scale SFE can be successfully transferred to pilot scale [39].

Major advantage of using supercritical CO_2 as solvent over traditional steam distillation process is in the quality of extract: extract obtained by steam distillation is blue oil containing mainly chamazulene and with traces of matricine (less then 0.1 mg/g), while the SFE extracts contains only matricine (up to 28 mg/g). These results suggest that chamomile flower heads do not contain chamazulene. Chamazulene, which is formed thermally from matricine, as its degradation product was not present in the SPF extracts or was found only in traces.. Chamazulene, the degradation product of matricine was detected only in the extract obtained by steam distillation. The content of matricine in SCF extracts was generally much higher than in conventional extracts. It can be concluded, that SFE offers considerable advantages over the conventional method of steam distillation and maceration for separation of essential oil compounds from chamomile [39].

10. SUPERCRITICAL FLUID EXTRACTION OF EUCALYPTUS LEAVES

Eucalyptus globulus is known for its richness in bioactive compounds such as essential oils, phenolic acids, flavonoids and hydrolysable tannins. Because of their multiple biological activities essential oils of *E. globulus* are used in medicine, perfume and food industry [40].

Supercritical fluid extraction was successfully applied to extract oil from Eucalyptus leaves using CO_2 and the effect of operating pressure, temperature and extraction time on the oil yield were studied using a central composition design methodology. Oil yield increased with increasing pressure and extraction time. However, temperature had a moderate quadratic effect and interacted with the pressure effect. A simplified regression equation was developed to predict the Eucalyptus leaves oil yield and was shown to be sufficient to describe the extraction process. The oil yield and chemical compositions of the oils obtained by SFE, hydrodistillation and Soxhlet extraction were also compared. In the Soxhlet extraction, the solvent type had a more significant effect on the oil yield than the extraction time, while in hydrodistillation, the oil yield increased with extraction time. The hydrodistillation gave the lowest yield of 3.77% while the ethanol Soxhlet extraction provided the highest yield of Eucalyptus leaves oil at

about 36.33%. It was also noted that due to the different mechanisms of the three methods, hydrodistillation had only extracted the volatile compounds, while Soxhlet extraction and SFE had extracted a wide range of components including not only the volatile but also the high molecular compounds. The content of 1,8-cineole in the extracted oils were 46.19% for SFE, 70.03% for hydro-distillation sample and 29.85% for ethanol Soxhlet sample. The addition of ethanol as a modifier in supercritical CO_2 improved the efficiency of supercritical fluid extraction. The yield of Eucalyptus leaves oil increased with increasing ethanol concentration. The compositions of the oil also differed quantitatively at different ethanol concentrations at low extraction pressures. There was a significant decrease in the 1,8-cineole content with increasing ethanol concentration from 5 w% to 15 w% at 10 MPa [41].

The other research performed with eucalyptus demonstrated the differences that exist between the composition and functional properties of extracts obtained with SFE (using supercritical carbon dioxide at 200 bar and 50ºC) and hydrodistillation. Antioxidant activity was found to be higher for supercritical fluid extracts than for hydrodistillation extracts. In spite of the fact that the main compounds were the same in extracts, their quantitative composition changed. For example, supercritical fluid extracts had a higher content of sesquiterpenes and oxygenated compounds. As for the antioxidant activity, it seems to be related to the concentration of both, p-cymen-7-ol and thymol. The concentration of both compounds, mainly the amount of p-cymen-7-ol, on SFE extracts was higher than that found on hydrodistillation extracts. Those values were in agreement with the higher antioxidant activity found in supercritical extracts compared to extracts from hydrodistillation [12].

11. LIPOSOMAL MICROENCAPSULATION USING SUPERCRITICAL FLUID PROCESSES

Liposomes are self-assembled spherical vesicles with one or more phospholipid bilayers separating the inner aqueous environment from the outer aqueous medium. The compound of interest was securely encapsulated in the inner aqueous core or within one or more phospholipid bilayers. They have shown great potential to act as a topical delivery system for carrying drugs and skin care products. Liposomes can transport drugs (cosmetic actives-CA) to target sites and maintain a higher drug (CA) concentration than conventional forms. As a result, the effectiveness of liposomal CA (drugs) can be enhanced for several folds. Because of the similarity in lipid composition to the epidermis, liposomes can also enhance dermal and transdermal drug delivery while reducing systemic absorption [38, 42–45].

Supercritical fluid processes for liposomal microencapsulation have drawn significant attention because the usage of organic solvent is substantially reduced or avoided, which results in less or even no pollution to the

environment. More importantly, this green technology provides pure encapsulated bioactives to consumers with no harm of toxicity [42, 43].

Traditionally, liposomes are prepared by thin-film hydration (TFH, the Bangham method), reverse phase evaporation vesicles (REV), and membrane extrusion. Nowadays, due to the concern of organic solvent toxicity, several new techniques have been attempted to reduce or even completely avoid the use of organic solvents in the liposomal microencapsulation, including microfluidics, rapid expansion of supercritical solutions (RESS), supercritical reverse phase evaporation (SCRPE), and several dense gas processes. Supercritical carbon dioxide (SC-CO_2) is a non-toxic density-adjustable fluid with the solvent behavior similar to hexane. Its moderate critical pressure (7.4 MPa) and low critical temperature (31.1^{o}C) make SC-CO_2 an ideal candidate for biomaterial processing [42].

Rapid expansion of a supercritical solution with continuous cargo suction microencapsulation (RESS-CCSM) was proposed as a non-toxic and continuous method for the formation of liposomal vesicles using SC-CO_2 as the sole phospholipids dissolving agent with the continuous cargo suction. Liposomes were reported to be successfully produced by a novel, non-toxic, and continuous technique of RESS-CCSM. The technique adopted and combined supercritical fluid extraction (SFE), RESS, microencapsulation, and vacuum driven cargo suction. SC-CO_2 was used as the sole dissolving agent for the coating material, soy phospholipids and cholesterol, without the involvement of organic solvents. The soy phospholipids and cholesterol blended in a mass ratio of 10:1 were loaded in the mixing vessel, followed by the introduction of SC-CO_2 up to 12.4 MPa at 60^{o}C. After two hours of phase equilibrium, the phospholipids/ /cholesterol-laden SC-CO_2 was directed into the expansion nozzle and simultaneously mixed with the TRIS buffer (pH 7.4) dissolving fluorescein isothiocyanate (FITC). The liposomes were instantly formed inside the nozzle during the CO_2 depressurization and directly collected in the glass tray for characterization. Under a confocal laser scanning microscope (CLSM), it was observed that hydrophilic substance was encapsulated in the liposomes. The liposomes produced using the RESS-CCSM technique were found to be mainly large unilamellar vesicles (LUVs) and multivesicular vesicles (MMVs) with the liposomal size less than 20 μm. The liposomes displayed good emulsion stability based on their high surface charge and low aggregation after 14 days of storage at 4^{o}C. The RESS-CCSM technique was claimed to be a non-toxic and continuous method for rapid production of liposomes [42].

Supercritical reverse phase evaporation (SCRPE) is similar to the conventional REV method for liposomal microencapsulation. The only difference is that SC-CO_2 is substituted for the organic solvent as the solution medium. It is a batch process and SC-CO_2 is used as the sole phospholipid-dissolving agent [42].

The DESAM process (depressurization of an expanded solution into aqueous media) was developed for bulk liposomal microencapsulation featuring

the reduced solvent residue of less than 4% (v/v) and moderate operating pressure between 4 and 5.5 MPa. The DESAM system mainly consists of the expansion and vesicle-generating vessels. 1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) and cholesterol in the molar ratio of 9 to 1 were dissolved in ethanol or chloroform. This solution was then injected into the expansion chamber followed by pressurization of CO_2 up to 4 and 5.5 MPa for chloroform and ethanol, respectively. During this step, the solution in the chamber was expanded with rising pressure. 10 min were required for the ternary phase equilibrium of the lipids, SC-CO_2, and solvent. Aqueous solution containing pure water or TRIS-buffered saline solution (20 mM TRIS; 0.9% NaCl and pH 7.4) was placed in the vesicle formation chamber (VFC) and heated up to 75^{o}C for liposomal formation. The authors also claimed that DESAM is a fast and simple process for bulk production of unilamellar liposomes [42].

The critical fluid nanosome (CFN) process – The injection and decompression techniques were the first two methods in success of liposomal microencapsulation using a supercritical fluid. The results suggested that the size distribution of the nanosomes varied from 100 nm to 4 mm. The majority of the nanosomes was less than 300 nm. No significant size change and bacterial growth were observed for the nanosomes made of egg yolk phosphatidylcholine (EPC) after six months of storage at 4^{o}C. The latter observation might reflect the capability of the CFN process to impart end-product sterility [42].

Particles from gas saturated solutions (PGSS) was initially designed for producing polymeric particles or encapsulating bioactive compounds in polymeric capsules. In this process, polymers and targeted bioactive compounds are first dissolved in a solution in an autoclave at a given temperature. SC-CO_2 is then introduced into the solution until the saturated concentration is reached. The SC-CO_2 saturated solution containing polymers and bioactive compounds is subsequently sprayed through a nozzle into a precipitation chamber. The microcapsules are formed and dried due to the intense cooling effect and solvent evaporation during the rapid depressurization of SC-CO_2 [42].

12. CONCLUSION

Supercritical fluid technology is now one of the major tools for processing food, cosmetics and pharmaceutical ingredients. The technology enables many key advantages, such as:

- Innovative products: - New food ingredients different from those obtained from classical solvent extraction or distillation and new pharmaceutical/cosmetic active ingredients can be achieved via synthesis, extraction, purification or formulation. Products demonstrate higher performance characteristics and purities, and contain no residual solvents;

- Regulatory and safety benefits - CO_2 serves as an alternative for organic solvents that are potentially toxic and shall be progressively banned for use in food and pharmaceutical products.
- Quality considerations - Supercritical technology allows developing high value products with new properties. Moreover food supplements and nutraceuticals keep their natural character or for phyto-pharmaceuticals/ cosmeceuticals moving from chemicals to natural processing.

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SUPERCRITICAL FLUID CHROMATOGRAPHY IN FAT-SOLUBLE E VITAMIN ACTIVE COMPONENTS ANALYSES OF PLANT NATURAL PRODUCTS

Summary

Vitamin E analogues have always been the subject of the researches throughout the years. The paper discusses the advantages of using Supercritical Fluid Chromatography (SFC) in analysis of vitamin E (and its analogues) and a comparison of this technique to other analytical methods. The attention is put on the applications of SFC, especially in vitamin E active components such as α-, β-, γ- and δ- tocopherols and tocotrienols analogues, and also other non-glyceride components analyses. However, still applications of modern packed column SFC with sub-µm particle in natural components analyses are in progress.

1. INTRODUCTION

Vitamin E is one of the groups of fat-soluble vitamins, next to vitamin A, D and K, including four tocopherols, such as α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol and four tocotrienols analogues, respectively α-T_3, β-T_3, γ-T_3 and δ-T_3, each of which has a chroman ring head in its structure [1, 2]. Moreover, the structures of these isomers differ in term of the methyl groups position in the mentioned ring [3]. Tocopherols and tocotrienols, which are referred to as tocochromanols, undergo the same biosynthetic process with the difference in hydrophobic tails saturation degree. Among all eight analogues of vitamin E, α-tocopherol is characterized by the highest activity [4, 3]. Figure 1 presents the basic difference in the structure of the tocopherols and tocotrienols.

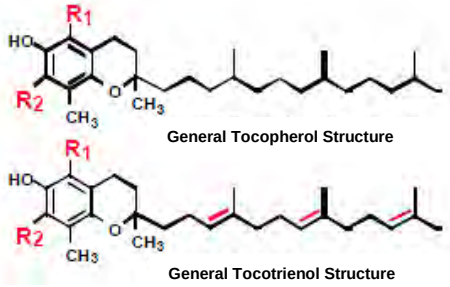

Fig. 1. The general structures of tocopherols and tocotrienols [4]

Tocopherols are obtained especially from plants and oilseeds and the characteristic feature of which is an anti-oxidation and nutraceutical properties, which are commonly used in food and dietetic products. Moreover, tocopherols are also added to cosmetics due to a skin damage prevention [2]. The high total content of tocopherols has been studied in wheat germ oil, soybean seed oil, corn seed oil and sunflower oil respectively [5, 6]. However, the greatest quantity of α-tocopherol varying between 90–96%, was analyzed in potato tubers, arabidopsis leaf but also in sunflower oil [5, 6]. Hess (1993) but also Taylor and Barnes (1981) in their studies focused on the analyses of tocopherols in lettuce leaf, spinach leaf, wheat seeds, rice (white grains) and corn seeds, in which different amounts of different vitamin E isomers were present. It has to be taken into consideration that the influence on both sample preparation and analytical separation has the fact that tocopherols may be rapidly degraded due to pH, light, oxygen as well as heat [2]. The presented study draws also attention on the degradation of the tocopherols and tocotrienols during the analyses in the contact with capillaries.

Vitamin E analogues have always been the subject of the researches throughout the years. Supercritical Fluid Chromatography (SFC) with the fluid in a supercritical state, has found successful and effective applications in both vitamin E analytical separation as well as fraction collection, which is to be discussed in the further parts of this study with the indication of different used solutions. What's is more interesting, SFC is also beneficial in the analyses of the minor components of plants extracts, such as carotenoids, lutein, triglycerides as well as other fat-soluble vitamins.

2. SUPERCRITICAL FLUID CHROMATOGRAPHY

The first Supercritical Fluid Chromatograph was equipped with a capillary column. However, due to impossibility in optimizing pressure and flow simultaneously in such mode, packed columns for SFC were introduced, taking into consideration the control of flow and pressure separately, which allowed to

use gradient but also modifiers and additives [2]. As far as Supercritical Fluid Chromatography (SFC) is concerned, it is a separation technique with the possibility of working both in a reversed phase and normal phase form. Moreover, Supercritical Fluid Chromatographs are equipped with similar modules as in a traditional high pressure liquid chromatography (HPLC). However, the characteristic feature of SFC is CO_2 in a supercritical state used as the main component of the mobile phase [9, 10]. Another aspect is that when working with a compressed CO_2, a backpressure regulator is necessary in order to keep a mobile phase under particular conditions [9, 10]. Similarly to a gas chromatography, in SFC the lower viscosity is, the higher flow rates are possible, which makes the analyses in SFC faster when compared to liquid chromatography. Figure 2 presents the differences of the three main chromatography techniques taking into consideration the changes in mobile phase.

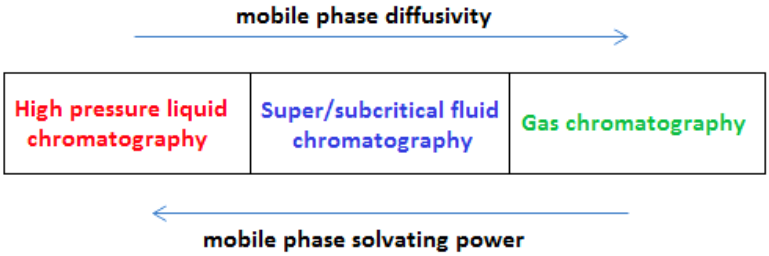

Fig. 2. The main chromatographic techniques differences in terms of the mobile phase [15]

In comparison to other chromatographic techniques in terms of particular parameters control, all three parameters, such as pressure, temperature and modifier are possible to be controlled in SFC, whereas GC does not allow to control pressure and modifier. As far as LC in concerned, the only pressure cannot be controlled during the analyses (Table 1) [7].

Table 1. Parameters under control in particular chromatographic technique [7]

Parameter under control	LC	SFC	GC
Pressure	-	+	-
Temperature	+	+	+
Modifier	+	+	-

It is stated that SFC is advantageous because of, for instance, fast equilibration, repeatability and water-based samples injection, not mentioning the lower costs of the analyses [9, 10].

2.1. Theory on chromatographic retention

The separation of the substances from the injected sample between the mobile and stationary phase is dependent on the partition coefficient of the particular analyte. Additionally, Van Deemter equation (1), which illustrates the dependence of the particular parameters on the height equation to a theoretical plate, is reflected in Supercritical Fluid Chromatography.

$$H = A + \frac{B}{v_0} + C_m v_0 + C_{st} v_0 \tag{1}$$

where A stands for Eddy diffusion, B stands for longitudinal diffusion of the analyte in the mobile phase, C_m stands for the velocity gradient in the mobile phase and C_{st} stands for the interactions of the injected analyte with the stationary phase. As it is stated, there is a particular point, in which the height equivalent of a theoretical plate (H) is optimal, according to a specified flow rate. Simply speaking, at a certain conditions of a flow rate, the column reaches the highest possible number of theoretical plates (N). Another crucial parameter describing chromatographic retention is resolution (Rs), which indicates the separation of the two closest peaks [2].

$$R_s = 1/4 N^{\frac{1}{2}} (\frac{\alpha - 1}{\alpha})[\frac{k}{k+1}] \tag{2}$$

in which N is the number of theoretical plates, α is the separation selectivity and k is the retention factor.

2.2. Crucial parameters affecting retention in SFC

There are at least three groups of crucial parameters which have the influence on the retention in Supercritical Fluid Chromatography. The first group consists of the parameters which concern the solvent strength of the supercritical fluid. The second group of the parameters focuses on the peak width during the elution. Lastly, the interaction of the substance with a stationary phase should also be taken into consideration [9].

In the case of temperature and pressure, both parameters affect the solvent strength of the supercritical fluid [2]. As it is stated, when a phase in enriched with a modifier, the pressure seems to result in lower retention as well as selectivity [9].

If it comes to the influence of the flow rate on the retention, it is sad that SFC is different when compared to HPLC in this regard. Namely, when the flow rate becomes higher, the pressure drop occurs on the column and as a consequence of this situation, the pump works under higher pressure as the back pressure remains unchanged [9].

The addition of the organic solvents, called modifiers to non-polar CO_2 has the influence on the changes in CO_2 polarity and density. The more solvent used, the higher CO_2 polarity is [2]. The reason for better separation with the use of the modifier may be caused by the deactivation of the stationary phase and the increase of its solvation [11]. Moreover, changes occur also in the retention due to the particular interactions. Such interactions include, for instance, interactions between dipoles or hydrogen bonds formation. However, if the content of the modifier is too high, the phase may possibly be separated into gas and liquid phase. In this case, the acceptable content of the modifier should not be lower or higher than 5–10% [8]. The most common modifier is methanol. Among other solvents there are also ethanol, acetonitrile, acetone, acetic acid, and many others acceptable [12].

2.3. Sample introduction

Different sample injection modes are possible, including direct injection, dynamic split injection, delayed split injection and finally timed-split injection [13]. In the case, where the entire volume is to be injected, a direct injection mode is appropriate. However, it does not allow to inject large volumes. Dynamic split injection is similar to delayed split injection with the difference that delayed split injection has an additional open/close valve, which closes when the initial injection is performed and then opens. Timed-split injection is a mode, in which the characteristic feature is a connection between the column and the injector valve [2, 14].

2.4. Chromatographic separation

In order to separate analytes with the use of SFC, the choice of the chromatographic columns is under the great importance. When polar analytes are to be analysed with the pure CO_2 it is preferable to work with open tubular columns (OT-SFC). However, these columns are limited in terms of sample capacity when large volumes of the sample are to be injected. In this case, packed columns (PC-SFC) are used, which allow faster analyses that the previously mentioned columns. In the analyses of the vitamins which are soluble in fats, fused silica OT-SFC columns are used as well as polysiloxanes with different chemical groups, such as methyl, octyl, cyanopropyl, phenyl and Carbowax [2, 16]. As for PC-SFC, the most common are the columns which are filled with porous silica or modified silica with octyl, octadecyl, cyanoalkyl and aminoalkyl groups [2]. Table 2 presents the examples of the applications of SFC in different compounds analyses taking into consideration different detection as well as type of stationary phase and the content of the mobile phase.

Table 2. Applications of SFC examples according to different stationary phase, mobile phase and detection

Compound	Stationary phase	Mobile phase	Detection	Reference
EPA, DHA	octadecyl silica or silica	pure CO_2	UV	[17, 18]
tocopherols, carotenes, sterols, squalene	silica	CO_2 + ethanol		[19, 20]
polyphenols	2 diol columns	CO_2 + methanol		[21]
tocopherols	silica + 10% polyethylene glycol	pure CO_2	MS	[22]
carotenoids	silica / bonded phenyl/ ODS	CO_2 + methanol with ammonium formate additive		[23]
carnosic acid, carnosol	silica + SE-54	pure CO_2	FID	[24]
underivatized amino acids	diol + silica	CO_2 + methanol + additives	ELSD	[25]
caffeine, monosaccharides	silica + ethylpyridine	CO_2 + methanol		[26]
triterpenoids	silica + ethylpyridine + phenyloxy propyl			[27]

2.5. Detection

There are at least three basic methods of detection used in Supercritical Fluid Chromatography, including UV-Vis, MS and FID. As compared to other methods, UV-Vis is applicable in SFC equipped with packed columns, whereas FID detector requires the use of open tubular columns and MS detector is commonly used with micropacked columns. The most selective and sensitive among all detectors, is fluorescence detector. However, it is used less commonly in SFC due to the possibility of losing selectivity when connected with high-pressure cells [2]. Moreover, in the case when the addition of the entrainer is too high, the changes in wavelength is observed. The more, for instance, methanol is added as the mobile phase, λ_{max} is shifted to higher values [28].

3. APPLICATIONS OF SFC IN VITAMIN E ACTIVE COMPONENTS ANALYSIS OF PLANT NATURAL PRODUCTS

A group of lipophilic bioactive vitamin E natural components, tocopherols and their unsaturated isomeric relatives α, β, γ and δ- tocotrienols have been

widely separated and quantified using Supercritical Fluid Chromatography. Previous studies on SFC separation of **individual tocopherols species** have been reported using both, capillary [31, 32] and packed columns [22, 34]. The first approach have been achieved with neat carbon dioxide and polar stationary phases enabling the separation of each tocopherol analogue since separation according to chain length or molecular weight with usage of nonpolar SFC columns was inaccurate for both β- and γ-tocopherols differing with the position of one methyl group [32, 35].

SFC separation of tocopherols using neat carbon dioxide can be highly improved when capillary column packings are coated with stationary phases normally used in gas chromatography (GC) and such procedure finally adjust polarity and selectivity of obtained package [36, 37]. Additionally, smaller internal diameters of columns filled with coated package particles characterized by smaller diameter have higher efficiencies allowing to achieve satisfactory separation of all tocopherol analogues (resolution at the baseline) and reliable quantitative results [37]. For polar stationary phases, coatings prepared with 95% methyl- 5% phenyl-silicone (SE-54) and 50% methyl- 50% phenyl-silicone (OV-17) with further crosslinking was recommended [36, 37]. However, the non-polar stationary phases both, octadecylsilica (C18) and SE-54 were unsuccessful separating β /γ tocopherol analogues [36].

Considering direct introduction of vegetable oil samples containing target compounds with large chemical nature at a very different concentration levels, especially such minor components as tocopherols, tocotrienols or other fat-soluble vitamins and phytosterols, quinones, squalene or carotenoids dissolved in a mixture of triglicerides (95–98%), the most important is the influence of the injected volumes and the concentration of the solutes (sample capacity and loadability) on the separation power of column [37]. The advantages of packed capillary SFC with greater diameter columns (up to 500 µm) with silica particles of package (10 µm ODS) coated with polyethylene glycol (Carbowax 20M) were successfully applied for direct coupling with an SFE extraction [37]. The method was optimized and revealed to be effective for extraction and fractionation of α, β, γ and δ- tocopherol analogues from test mixture without losing resolution. However, the recovery was not higher than 90% in the case of α-tocopherol. Model mixtures of carotenes and sterols have also been separated by SFC, but in real sample matrix interferences from coexisting compounds have strong influence on separation and retention of target compounds.

Separations of minor constituents from natural plant oils became under the great importance in terms of their various beneficial properties including antioxidant, anticarcinogenic and hypocholesterolemic potential [39, 40, 41, 42]. Tocopherols and tocotrienols can be effectively concentrated either from the palm fruits oil (crude palm oil CPO) or palm pressed fiber oil (PFO) and isolated as a form of pure fractions by SFC. PFO is enriched much more with minor components such as vitamin E (2000–4000 ppm), carotenoids (4000–6000 ppm) and sterols (4000–6500 ppm) when compared to the crude one [43, 44].

A thorough study dedicated to SFC of active components of vitamin E is the one provided by Choo and co-researchers. These investigations concerned both SFC analytical separation and preparative fractionation. The first approach was performed with the use of silica column and CO_2 with ethanol (4%) as mobile phase resulting in separation of five tocopherol and tocotrienol analogues with the elution order of: α-tocopherol, α-tocotrienol, γ-tocopherol, γ-tocotrienol and δ-tocotrienol [43]. In study by Ng et al., the same effect on the separation of all active natural components of vitamin E form palm oil was studied on diol column and the elution order of mentioned analytes was confirmed with eight-component standard of tocopherols and tocotrienols (α-T, α-T3, β-T, δ-T, β-T3, γ-T, γ-T3, δ-T3) [45, 46]. At modifier concentrations of 4–6%, two mobile phases were composed of CO2- EtOH and CO2 – methyl-tert-butyl-ether and used for the separation with silica and diol columns respectively [47].

SFC coupled with UV-variable wavelength UV/vis detector was proved to be more straightforward and less time-consuming than other analytical methods (HPLC, GC, UV-spectrophotometry), in isolation and quantification of such minor components of palm oil (CPO and PFO) as carotenes, vitamin E, sterols and squalene [43]. Column packed with 5 µm silica (25 x 0.46 cm) provided satisfactory separation performance of all mentioned minor components within quite a short time comparing to HPLC or GC. The last group of sterols, guantified as β-sitosterol, were found eluting at 12 minute, whereas the elution order of remaining earlier eluting target compounds was squalene, carotenes, vitamin E [43]. The quantitative results of squalene obtained by SFC analysis was comparable to those obtained with gas chromatograph-FID. Similarly concentrations of carotenes, calibrated as total β-carotene the major one in palm oil, analyzed by SFC were in good agreement with UV-Vis spectrometry results. As compared to HPLC, SFC analyses gave higher concentrations of palm oil tocopherols and tocotrienols analogues typically found in palm oil that was clearly shown in the case of γ-tocotrienol and δ- tocotrienol (results in SFC were higher by 50 ppm and 40 ppm respectively). This difference was attributed to the fact that tocopherols were susceptible to the higher exposition to organic solvents used with HPLC. However, tocotrienol standards, especially δ- tocotrienol, were previously revealed to be more prone to oxidative damage than tocopherols [48, 49]. The elution order of tocopherols and tocotrienols analogues from palm oil was typical for normal stationary phase [43, 48].

Tocopherols separation is successfully accomplished in modern SFC systems enabling the use of packed high performance chromatographic columns and, depending on the polarity of stationary phase, work in the normal phase mode [34] or reversed phase mode [37]. Thus, with independent control of flow and pressure enabling the use of mobile phase gradients in the system, temperature and outlet pressure kept at constant critical conditions, the separation can be performed either by changing stationary phase or mobile phase polarity. The last mentioned criterion for selecting the SFC system is mobile phase polarity adjusted by the addition of precise content of polar modifier

affecting density and linear velocity of CO_2, which consequently varies the analytes retention. The lower viscosity, the highest number of peaks can be separated and longer and more efficient columns can be applied [8, 50]. Yarita et al. examined the retention behavior of tocopherols on ODS-silica gel column depending on the modifier concentration in the mobile phase [52]. The low methanol concentrations (0.5%) blended with CO_2 produced a mobile phase enabling baseline separation of β- and δ- tocopherol homologues with the highest resolution. Further increase of the modifier content resulted in decreasing both retention factor and resolution between isomers.

When choosing stationary phase of chromatographic package, mobile phase composition needs to be taken into consideration. The most commonly used stationary phase compatible with SFC is spherical porous silica (particle sizes 3–10 μm in diameter, pore sizes 100–300 Å) [2]. Different retention modes can be obtained with a particular stationary phase, especially bonded with different chemical groups of compounds, octyl, octadecyl (ODS) which enable differing solutes by their methyl and methylene groups and diol, aminopropyl, cyanopropyl and polyethylene glycol bonded silica as polar stationary phase [8].

Another field of application for packed column SFC is preparative scale purification of unsaponifiable compounds, bioactives (e.g. fat-soluble vitamins) that can be of a special interest in food, cosmeceutical and even pharmaceutical industries. Considering some crucial advantages offered by prep-SFC, such as faster column equilibration and higher resolution per unit of time, reduction in solvent consumption and easy analytes collection in a small volume of organic co-solvent after depressurization together with higher flow rates and better production rates, the technique has been found successful in separating, purifying and recovering palm oil vitamin E homologues [8, 53, 54]. The enrichment of tocopherols and other non-glyceride active components from different vegetable oils has been also performed by coupled SFE extraction and prep-SFC (Table 3).

Table 3. Application of supercritical CO_2 separation method in minor lipid compounds [55]

Component	Raw material	Technique	Reference
Ferulate Phytosterol esters	Corn bran oil	SFE + SFC	[56]
	Corn fiber oil	SFE + SFC	[57]
Tocopherols	Soybean oil Rice bran oil	SFE + SFC SFE + SFC	[58] [58]
	Wheat germ oil	SFC + SFE Semi-prep SFC	[58] [8]
Squalene	Olive husk oil	SFE	

Nowadays, the use of SFC continues to expand into application in the field of ultra-high performance supercritical fluid chromatography in which sub-2 µm fully porous particles in column package can be successfully used [8]. When applying binary mobile phase composed of CO_2 and modifier (most often methanol or ethanol) blended in small proportions (2–30%), such features as eluent strength and analyte diffusion coefficient can be adjusted enabling analyses of either lipophilic compounds or molecules with a broad range of a polar character. However, the available publications concern pharmaceutical compounds such as steroids and benzodiazepines [51]. Further studies, especially on natural products are still in progress. The only available are Waters application notes with their UPC2 system (Ultra-Performance Convergence Chromatography) and packed columns with sub-2 µm FPP stationary phases with different chemistries.

Separations of all eight tocochromanols under RP-UPC2 was performed with HSS C18 SB (10 x 3.0 cm) and 1.8 µm particle size (own researches) with methanol as co-eluent. The solutes were separated at baseline as depicted at Figure 3.

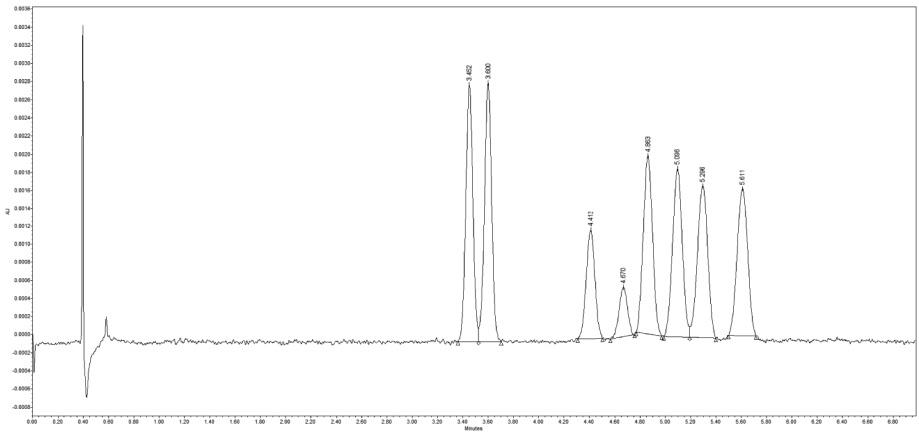

Fig. 3. UPC2 analysis of vitamin E active components (elution order : α-T, α-T3, β-T, δ-T, β-T3, γ-T, γ-T3, δ- T3)

4. CONCLUSIONS

Supercritical Fluid Chromatography (SFC) is an important separation technique in the analyses of a broad range of analytes. The main, thus the beneficial aspect of SFC is the ability to combine different properties of both HPLC and GC [5, 30]. The development of this technique through the past years offers new opportunities and solutions [5]. There are at least several advantages of SFC over HPLC, which allows SFC to take a place before HPLC in this aspect. First of all, SFC is faster method than a traditional liquid

chromatography, on the account of the characteristic features of SFC mobile phase, including high diffusivity and low viscosity in the comparison with HPLC. Secondly, such mobile phases are applicable in Supercritical Fluid Chromatography equipped with mass spectrometer. SFC is also beneficial in terms of the low costs, as the solvent consumption and solvent disposal are reduced in such mode. Moreover, even with the addition of modifiers, such as methanol or other alcohols, CO_2 is more environmentally friendly than the solvents used in both normal phase and reversed phase HPLC [29].

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SC-CO_2 *SPIRULINA PLATENSIS* EXTRACT AS PLANT GROWTH BIOSTIMULANT

Summary

The effect of *Spirulina platensis*-based formulation on *Cucumis sativus* was investigated. Formulations, optionally containing micronutrients, were tested in pot experiments after foliar applications at 3 field dose-corresponding concentrations. For comparison purposes, groups treated with water (negative control), mixture of co-formulants (positive control), and reference products were also evaluated. Most treatments increased dynamics of gas exchange and fruit mass, while only two *Spirulina platensis*-based working solutions affected additionally both length and diameter of fruits. Supplementation with micronutrients did not enhance activity of algal formulation, although it generally exceeded commercial stimulants.

1. INTRODUCTION

1A. Scope of research

The present paper focuses on applicability of new naturally-based product for stimulation of plant growth and condition. Since current agriculture faces insufficiency in crop production, while legislation tightens the use of fertilizers

and crop protection products, improvement of conventional treatment is needed. For the last two decades, one can observe a tendency to combine mineral fertilizing with application of biological materials capable of promoting natural plant ability to both growth and effective nourishment [1]. Such materials – defined under common name "biostimulants", include substances and/or microorganisms which in low doses [2] "enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality" [3]. Among known biostimulants, algae and algae-based products – mainly extracts, are of the great interest [4]. Although seaweeds have been proved for their phyto-stimulatory activity [4, 5], there are still a few reports on the use of microalgae biomass in plant cultivation.

Microalgae are considered as extraordinary – compared to terrestrial plants, source of fatty acids (FAs), which might be the component of food and feed additives, biofuels and high-value products [6]. It was shown that extracting microalgal biomass with supercritical fluids – supercritical CO_2 in particular, provided high yield of fatty acids, including ω-3, ω-6 and ω-9 polyunsaturated fatty acids [7, 8]. In plants, FAs serve various functions in cell membrane synthesis, energy storage and response pathways, affecting development and whole-organism performance [9, 10]. Despite proven antioxidant and signaling activity of fatty acids [7,9], their use for plant growth stimulation is rather undertested.

The aim of present paper was to examine beneficial effect of formulation containing supercritical CO_2 extract from *Spirulina platensis*, optionally enriched with micronutrients, in pot trials on cucumber. Plants were treated foliarly and examined for growth parameters of mature fruits.

2. POT TRIALS ON *CUCUMIS SATIVUS*

2A. Composition of *S. platensis*-based biostimulants

Low polarity of SC-CO_2 resulted in hydrophobicity of the extract which needs to be added to water-miscible formulation – such as emulsifiable concentration, enabling the provision of working solution. Preparation of supercritical extract from *S. platensis* powder (WB Im- und Export W. Beringer & Co. GmbH, Germany) and it-containing formulation – suitable for plant treatment, were performed in accordance with the procedures of innovation [11, 12]. There were two microalgae-based biostimulants formulated for the experiment, and one included micronutrients. To verify component-activity dependence for examined products, mixtures of constituents other than the extract – hereinafter defined as co-formulants, were also prepared. The complete composition of final formulations was listed in Table 1.

Table 1. The composition of *Spirulina platensis*-based formulations and mixtures of co-formulants; "+" mark corresponds to contained components, while "–" mark – otherwise

Component	CAS no.	Content [%, by mass]	Formulations non-enriched with micronutrients		Formulations enriched with micronutrients	
			Co-formulant mixture	Algae-based formulation	Co-formulant mixture	Algae-based formulation
1st phase						
SC-CO_2 extract from *S. platensis*	n/a	10.0	–	+	–	+
Emulsifier ETR 1247 (Croda Poland)	n/a	2.50	+	+	+	+
Emulsifier ETK 0794 (Croda Poland)	n/a	2.50	+	+	+	+
2nd phase						
Anti-freeze agent	25322-68-3	2.50	+	+	+	+
3rd phase						
Preservative	24634-61-5	0.0100	+	+	+	+
Boron	10043-35-3	0.0200	–	–	+	+
Copper	7758-99-8	0.0500	–	–	+	+
Iron	7782-63-0	0.100	–	–	+	+
Manganese	10034-96-5	0.0500	–	–	+	+
Molybdenum	12054-85-2	$1.00 \cdot 10^{-3}$	–	–	+	+
Zinc	7446-20-0	0.0500	–	–	+	+
Water	7732-18-5	to 100%	+	+	+	+

n/a – not applicable

The composition of formulation components was elaborated based on previous tests on crops under field conditions [unpublished data].

2B. Pot trials and growth/health measurements

Supercritical extract from *Spirulina platensis* owes its activity strongly to fatty acids [13] stimulatory effect of which was verified in pot trials on *Cucumis sativus* ("Śremski" cultivar). The experiment was conducted in a plant house – located on premises of University of Warmia and Mazury (Olsztyn, Poland), between April and June 2016. Cucumber seeds were sown and allowed to grow until one-month-old seedlings were planted singly in 9 L pot for further investigation (Fig. 1). For the experiment standard soil was selected. Cultivation conditions were, as follows: 16:8h photoperiod, light/dark phase temperature of $25^{o}C \pm 2^{o}C/19^{o}C \pm 2^{o}C$, air humidity at 70%±5% and 65% field water capacity.

Fig. 1. Pot trials on *Cucumis sativus* in a plant house at University of Warmia and Mazury; planting seedlings (top), post-treatment plants (bottom, left), pre-harvesting fruit (bottom, right)

On the 60th day of the experiment (30 days after planting seedlings), plants were subjected to foliar treatments, as shown in Table 2: six combinations of *Spirulina platensis*-based compositions, six combinations of co-formulant mixtures (positive control groups), two commercial biostimulants (reference groups) and water (negative control group).

Table 2. Schedule of treatments conducted on cucumber seedlings

Series (group)		Working solution [%, by volume]	Designation used in the paper
Product	Formulation applied		
Mixture of co-formulants	non-enriched with micronutrients	0.50 0.74 0.89	Co-F 1.0 Co-F 1.5 Co-F 1.8
	enriched with micronutrients: H_3BO_3, Cu^{2+},Fe^{2+}, MoO_4^{2-}, Mn^{2+}, Zn^{2+}	0.50 0.74 0.89	Co-F+MN 1.0 Co-F+MN 1.5 Co-F+MN 1.8
S. platensis-based formulation	non-enriched with micronutrients	0.50 0.74 0.89	SP-F 1.0 SP-F 1.5 SP-F 1.8
	enriched with micronutrients: H_3BO_3, Cu^{2+},Fe^{2+}, MoO_4^{2-}, Mn^{2+}, Zn^{2+}	0.50 0.74 0.89	SP-F+MN 1.0 SP-F+MN 1.5 SP-F+MN 1.8
Reference products	Asahi SL (Arysta LifeScience, USA)	0.10	Asahi
	Forthial (Goëmar, France)	0.25	Forthial
Water	–	–	Control

No mineral fertilizing was performed. Applied concentration of prepared formulations corresponded to field-scale doses: 1.0, 1.5 and 1.8 L (in 200 L water) per ha [unpublished data]. Solutions of reference products complied with the guidelines for seedling supplementation. Each treated group included 2 replicates (2 plants).

The effect of foliar treatment on plant physiology and overall health was determined by measuring gas exchange and chlorophyll content. The evaluation of the growth of cucumber fruits was also performed for 30 consecutive days after product application.

Dynamics of gas exchange – including both net photosynthesis and transpiration rate (NPR and TR, respectively), and chlorophyll content in leaves – expressed by leaf greenness index (LGI), were assessed for each seedling 2 days after plant treatment. Gas exchange measurements were conducted on the youngest fully developed leaves, at constant CO_2 levels of 400 ppm, with LI-COR 6400 Portable Photosynthesis System (DMP AG SA Ltd, Switzerland). For examination of leaf greenness index Soil Plant Analysis Development (SPAD)-502 chlorophyll meter (Konica Minolta Co. Ltd, Japan) was used.

Cucumber fruits were harvested at the time they reach maturity and investigated for growth parameters such as: mass, length and diameter of a single fruit. Assessment of growth parameters was conducted for each harvest per a given group.

Measured values of growth parameters were elaborated with Statistica ver. 12 software (StatSoft, a part of Dell Software, USA). Among obtained data, distribution normality (Shapiro-Wilk test) and, if concerned, variance homogeneity (ANOVA; Brown–Forsyth test) were verified. The results of statistical analysis were presented in box-and-whiskers plots as means and medians for normally and non-normally distributed data, respectively.

Significance of differences between the entry groups, was confirmed at p value below 0.05 by either Tukey's honestly significant difference test or Kruskal-Wallis test among parametric and non-parametric data, respectively.

2C. Results and Discussion

Evaluation of both growth and health parameters proved tested formulations as safe-to-use at the range of doses applied since no visual phytotoxicity symptoms and/or local lesions were noted, compared to the negative control and reference groups.

Dynamics of gas exchange in cucumber seedlings was, generally, stimulated by foliar treatment, though the results of measured parameters were not correlated (Fig. 2). Most of applied products enhanced transpiration (8–55% over the negative control) rather than net photosynthesis rate (8–33% over the negative control). Efficacy of each treatment differed as either NPR or TR was taken into consideration, except Forthial – application of which provided one of the highest results for both parameters. Beside the extremes, obtained means were similar

among groups showing differences below 10%. Although no evident dose-response relationship was confirmed, there was a slight increasing trend observed for the results as higher dose of Co-F, Co-F+MN and SP-F+MN was applied. Activity of non-enriched *S. platensis*-based formulation toward gas exchange was, at the same time, inverse.

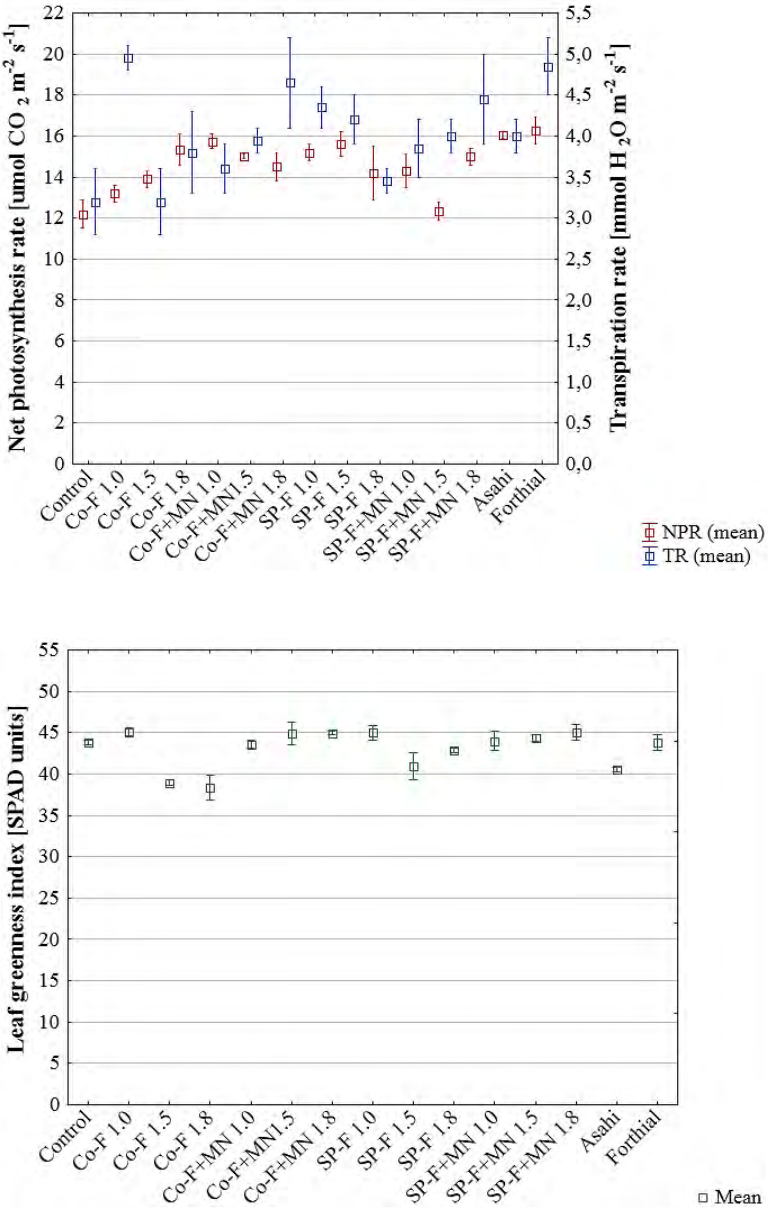

Fig. 2. Gas exchange dynamics and leaf chlorophyll content in cucumber plants; *whiskers* corresponds to *mean±standard error*

As opposed to gas exchange measurements, chlorophyll content was affected by neither microalgae-based nor reference products compared to both negative and positive control groups. The lowest LGI means (11–13% below the negative control) obtained for co-formulants mixture at 0.74 and 0.89% might resulted from device accuracy – which was ±1.0 SPAD units, and did not seem to disturb overall similarity of values obtained.

Since the results were at random, the effect size of examined treatments on gas exchange dynamics or leaf greenness was not determined undoubtedly. Considering dependence between overall plant condition and growth, health indicators did not correspond to measurements of biomass production.

In comparison with means of NPR, TR and LGI, average values of growth parameters were more diverse and ordered, and thus, influence of applied products might be evaluated more clearly (Fig. 3).

Foliar treatment mostly affected fruit mass, which was generally higher by 11–55% than the negative control. Diameter of cucumber fruits was enhanced (12–17% over the negative control) at the lowest and medium dose of Co-F+MN and SP-F, respectively; as well as by SP-F 1.8 (10%) and SP-F+MN 1.5 (12%). The latter two also provided the best results in fruit length (13 and 5% over the negative control, respectively). Considering overall fruit growth, *S. platensis*-based formulations showed the highest phyto-stimulatory activity and non-enriched product at 0.89% was the preferable treatment. Beneficial effect of reference biostimulants was observed only on fruit mass (15–16% over the negative control), though it was weaker compared to most efficient microalgae- and co-formulants-based solutions (39–55% and 44%, respectively). Under conditions of the experiment, supercritical *S. platensis* extract affected model plant growth stronger than filtrate from brown seaweed *Ascophyllum nodosum* (Forthial) or nitrophenols of phyto-origin (Asahi SL). Despite promising results, obtained data did not differ significantly among groups.

Based on growth measurements, it was also noted that addition of micronutrients strengthened the influence of co-formulant mixture on plants, while causing the opposite effect as *S. platensis*-based formulation was taken into account. On the other hand, enrichment products with micronutrients did not affect dynamics of gas exchange, except shift in the dose-related trend noted for SP-F. Such difference might result from various behaviour of biologically active compounds present in SC-CO_2 algal extract when applied along with other components and at a given concentration. Since neither the interaction biomolecules might be subjected to nor the mechanism according to which they would promote plant response are well-known [5], variations in activity of microalgae-based biostimulant require further investigation.

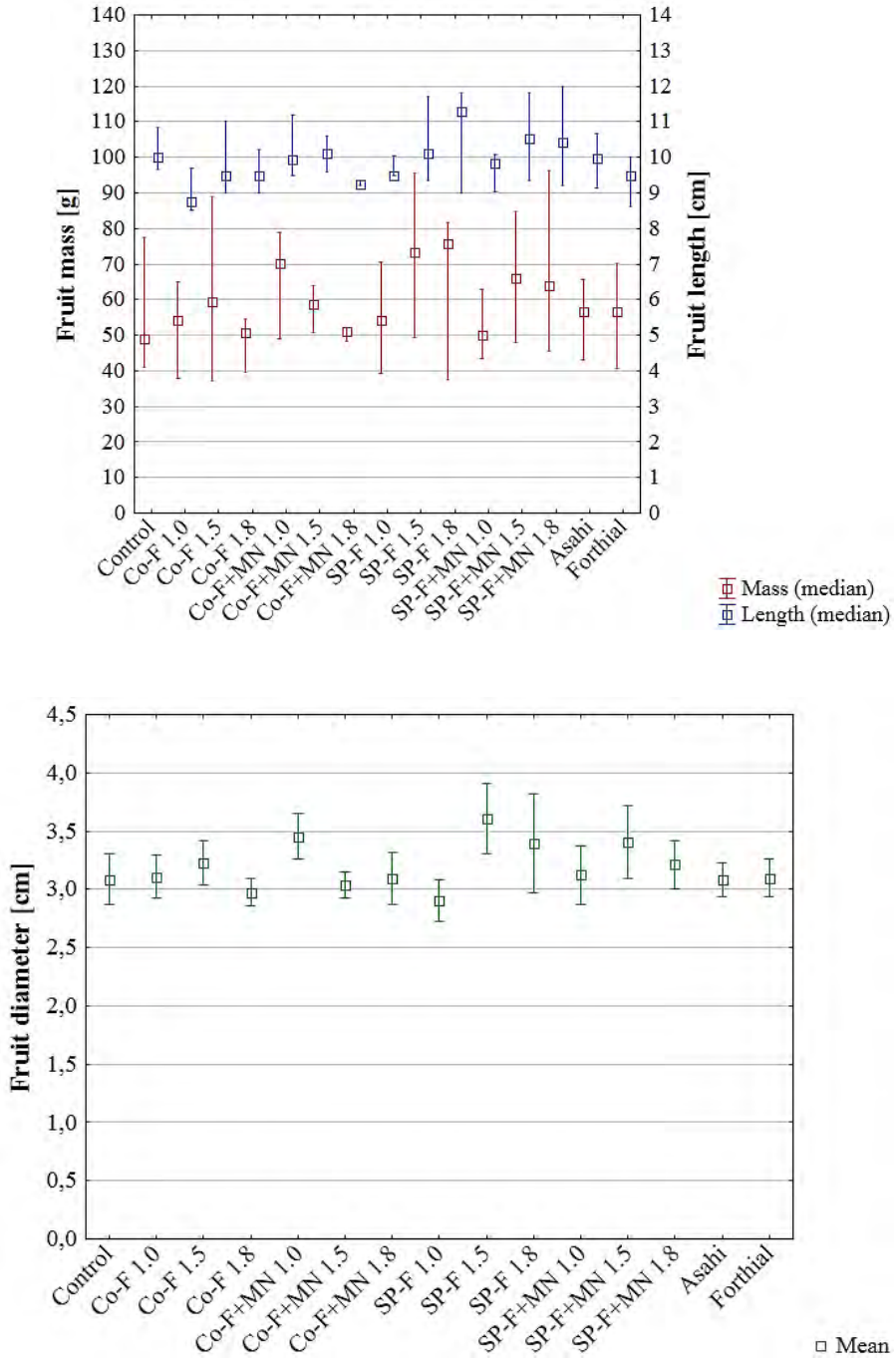

Fig. 3. Growth parameters of cucumber fruits; box corresponds to 25th–75th percentil (for median) or mean±standard error, while whiskers – non-outlier range (for median) or mean ± standard deviation

In terrestrial plants, metabolism of lipids and their inter-cell-layer communication and transfer – all are involved in overall growth regulation during whole life cycle of the organism [10, 14]. Mutation in specification of FA-containing epidermal cell might disorganize plant morphology [15–17], while inhibition of very-log-chain fatty acids (at least C20) synthesis has been shown to cause overproliferation of cell of various types [14]. At the same time, reports confirmed chain length-depending activity of FAs when applied externally to seeds. Treatment with short chain FAs (C6–C10) limited germination, wherein the disadvantageous effect increased at higher concentration (up to 10 mM). Contrariwise, long chain fatty acids provided the results comparable to the control [18–20]. Yet, there are no sufficient information about plant supplementation with FAs at late vegetative phase and/or by foliar spraying.

Determination of the fatty acid profile in SC-CO_2 extract from *S. platensis* showed main content (78%, in total) of palmitic acid (C16), linoleic acid (C18:2, ω-6) and linolenic acid (C18:3) [21], including γ-linolenic acid (ω-6) [22]. For aforementioned ω-6 fatty acids dietary and medicinal application have been preferably investigated [23], hence, their phyto-stimulatory effect is not well known.

Previously conducted laboratory-scale experiments verified supercritical *S. platensis* extract to promote sprouting – both rate and growth, of *Brassicaceae* plants (*Lepidum sativum, Sinapis alba* and *Brassica napus*) and cereal (*Triticum aestivum*). The influence of microalgae-based formulations on plant germination was observed regardless of application method – foliar spraying, seed coating, spilling to soil [24-26]; the former enhanced mostly mass production [25] confirmation of which was denoted in pot trials on *C. sativus*.

Efficacy of SC-CO_2 microalgal extract was also evaluated on winter wheat under field conditions and compared to the use of Asahi SL. As opposed to cucumber, *T. aestivum* growth increased the most at the lowest dose (1.2 L per ha) of *S. platensis*-based formulation surpassing the negative control and reference product in grain yield by 12% and 9%, respectively. Mineral composition of collected grains was similarly improved by each biostimulant, as well [28].

Considering cucumber as a model plant, its response to application of *S. platensis*-derived products has not been well-examined. There are reports on beneficial treatment of *C. sativus* with microalgae of different species, though research focused on overall plant growth rather than fruiting. Shariatmadari et al. (2011) performed both germination and pot trials to prove stimulatory effect of *Anabaena vaginicola*, *Nodularia harveyana* and *Nostoc* sp. when used as suspended in water. It was shown that soaking cucumber seeds in microalgae suspension enhanced germination rate and growth parameters providing significant increase in sprout height. Seedling height was also significantly affected by daily spraying of the suspension which, moreover, strongly influenced root growth (differing from the control at $p<0.05$). Among tested microalgae, *A. vaginicola* and *Nostoc* sp. showed comparably high activity while for *N. harveyana* weaker effect was observed [29]. Applicability of

A. vaginicola to cucumber treatment was also verified in the work of Riahi et al. [30]. The results reported by Shariatmadari et al. were in accordance with previous investigation of Nanda et al. (1991) concerning the influence of *Westiellopsis prolific* on cucumber at different vegetation phase [31].

Foliar application of microalgae has been proved to beneficially affect *C. sativus* in germination, overall seedling growth and fruit production, thus examination under field conditions is required to develop the procedure of efficient cucumber treatment.

3. SUMMARY

Stimulatory effect of SC-CO_2 extract from microalgae Spirulina platensis was confirmed on cucumber fruits under controlled conditions. It was shown that microalgae-based products had stronger influence on growth parameters compared to commercial stimulants. There is however a need to verify obtained results in field trials which will also include qualitative evaluation of the fruits.

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OPTIMIZATION OF METHODS OF FATTY ACIDS EXTRACTION FROM BIOMASS OF SCHIZOCHYTRIUM LIMACINUM

Summary

Schizochytrium limacinum Honda & Yokochi (Thraustochytriaceae) is heterotrophic microorganism, characterized by a high content of fatty acids, especially docosahexaenoic acid (DHA), which plays an important role in the proper functioning of cells and organs of mammals. The aim of this study was the comparison of various extraction methods, such as Soxhlet extraction, ultrasound-assisted extraction (UAE), microwave assisted extraction (MAE) and supercritical fluid extraction (SFE) for fatty acids recovery from biomass of *S. limacinum.* For obtaining fatty acids from *S. limacinum* biomass from extraction process of biomass most efficient extraction methods are SFE and MAE with the preliminary process of homogenization of the material.

1. INTRODUCTION

For pharmaceutical and cosmetic industry, extracts from plants, algae, fungi or microorganisms have valuable chemical compounds with high biological

activity. Acquiring these methods bioactive ingredients for cosmetic purposes is considered to be more attractive to consumers than the identical synthetic molecules. These compounds are used in the production of cosmetics called "line of natural origin". Effective obtaining bioactive compounds by extraction depend on many parameters. One is the ability to penetration of solvent through the membrane into the cells, which are located extracted bioactive components. A destruction of the structure of the outer membrane and the cell membrane of the microorganism in the initial stage preceding the extraction process determines the efficiency of extraction as well as determines the qualitative and quantitative composition of the extract. The problem of destruction of the structure of the outer membrane of the microorganism or cell membrane in the initial extraction stage is particularly important for heterotrophic microorganisms cultured in order to obtain bioactive compounds with specific properties e.g. unsaturated fatty acids.

Schizochytrium limacinum Honda & Yokochi (Thraustochytriaceae) is a heterotrophic micro-organism first isolated from the mangrove area in the west Pacific Ocean. It is classified into the kingdom *Chromalveolata* – super group of *Eukaryotes*, resulting secondary endosymbiont algae. This organism is attracting attention because of its high content of, especially docosahexaenoic acid (DHA, C22:6, n-3), which plays an important role in the proper functioning of the cells and organs of mammals [1–6]. DHA belongs to the group of omega-3 fatty acids, as well as to EFAs (Essential Fatty Acids), because the human body is unable to synthesize them itself. DHA is synthesized from α-linolenic acid, one of the most important essential fatty acids in human body. The best source of DHA is fish oil, although it is encountered in some algae or flaxseed. Docosahexaenoic acid occurs in the largest concentration in the phospholipids of the retina and in certain brain areas – it is particularly important here, because it affects the final shape of the nervous system and mental capacity [7]. It is very important for pregnant women to assist the DHA supplements, because the child's only source of this acid is its mother. Docosahexaenoic acid is primarily active ingredient in anti-aging, soothes and protects the skin against inflammation, acts photoprotectively and strengthens the immune system of the skin, preventing aging of the skin [8]. Biomass of *S. limacinumis* is used to production of biodiesel by direct transesterification [9].

The basic procedure for the isolation of substances from plant raw materials extraction is made using different methods [10, 11]. While in traditional herbal medicine is used aqueous or alcohol extraction by Soxthlet method. Increasingly are used unconventional methods of extraction, such as extraction using microwave [12–16], or extraction using ultrasonic field [17]. Solvent extraction assisted with ultrasound (UAE) consists of placing solid or semi-solid samples, together with the extractant in an ultrasonic bath or the use of probes generating ultrasound. Operation of the ultrasonic waves causes change in a pressure wave that significantly accelerates the extraction. A significant development in facilitating the extraction in this case is cavitation, or formation and

disappearance of vapor and gas bubbles, which bursts generate high pressure and temperature, which helps the penetration of the solvent. The advantage of this method are the short duration of the process, of from 10 minutes to 1 hour, the possibility of carrying out the process at room temperature, high solubility and diffusion of the solvent. The process entails also some disadvantages. The need to separate the extract from the resulting residue increases the energy consumption of the process, and cavitation may cause the reactive components, causing changes in the composition of the product obtained [18, 19].

Solvent extraction assisted microwave irradiation (MAE) is the process of using microwave energy absorption by chemicals to extract it from raw material. Material together with the solvent, the organic substances are placed in the vessel which generates microwave radiation, in which the temperature reaches up to 190°C. The energy affects the movement of ions and dipoles in the electromagnetic field, resulting in the generation of heat, which results in increasing process efficiency. The condition of solvent selection is its non-zero dipole moment. The effectiveness of this method of extraction is high considering short-time process, but the heat which could lead to decomposition of the product and the use of organic substances [20–22].

One of the ways the extraction of natural materials is a supercritical extraction. Supercritical extraction used as extractant normal gas at a pressure higher than its critical pressure and at temperatures above its critical temperature. This method has found wide application in the secretion of the extracts of vegetable raw materials particularly heat sensitive [23].

In this paper we presented results of biomass extraction by different method, and discussed influence of methods of biomass preparation in initial process on results extraction. The extraction efficiency and the composition in extract mixture of fatty acids were evaluated.

2. EXPERIMENTAL

S. limacinum was purchased in Alltech Ireland Ltd. All chemicals (Aldrich, POCH) used in the experiments, such as hexane was of analytical reagent (AR) grade. Microscopic photographs of the *S. limacinum* biomass were made by B. Messyasz using a light microscope (Zeiss Axioskop 2 MOT) under 400x and 1000x magnification.

The Soxhlet extraction

A) Without homogenization step

10 grams of dry powdered material of *S. limacinum* biomass was weighed out end extracted with hexane (200 mL). The extractions were performed at 25 cycles (8 h). After extraction, the resulting solutions was filtered to pre-weighed round bottom flasks and concentrated on a rotary evaporator at 40°C.

The extracts were weighed and yield was calculated according to the time of extraction. These extracts were stored at 4°C for analysis.

B) With homogenization step

10 grams of the raw material was transferred to a falcon tube and 20 mL of hexane was added. The solution was homogenized (ultrasonic homogenizer, 26 kHz, 200 W, Hielscher UP200HT) for 1 h. During the homogenization system was cooled on ice. The resulting mixture was placed in an ultrasonic bath (Cole-Parmer 8891) and sonicated for 30 minutes. Broken cells were transferred quantitatively into a thimble and extracted in a Soxhlet apparatus. The extractions were performed at cycles (8 h). After extraction, the resulting solutions was filtered to pre-weighed round bottom flasks and concentrated on a rotary evaporator at 40°C. The extracts were weighed and yield was calculated according to the time of extraction. These extracts were stored at 4°C for analysis.

Supercritical fluid extraction (SFE)

A) Without homogenization step

The extraction of *Schizochytrium limacinum* biomass (about 100 g) was carried out using the multi-purpose pilot unit for supercritical fluid extraction produced by SITEC/Switzerland. The extraction plant has an extractor of capacity of 1 liter, and can operate at pressures up to 1000 bar and temperature up to two 200°C. Two stage separation allows fractionation of the produced extract continuously. An extraction was performed at 700 bar and at 45°C. Pure CO_2 was used as solvent, and samples were taken every 30 minutes. These extracts were stored at 4°C for analysis.

B) With homogenization step

10 grams of the raw material was transferred to a falcon tube and 20 mL of hexane was added. The solution was homogenized (ultrasonic homogenizer, 26 kHz, 200 W, Hielscher UP200HT) for 1 h. During the homogenization system was cooled on ice. The resulting mixture was placed in an ultrasonic bath (Cole-Parmer 8891) and sonicated for 30 minutes. The hexane was evaporated. The extraction of *S. limacinum* biomass after homogenized process was carried out using the multi-purpose pilot unit for supercritical fluid extraction. An extraction was performed at 700 bar and at 45°C. Pure CO_2 was used as solvent, and samples were taken every 30 minutes. These extracts were stored at 4°C for analysis.

The microwave assisted extraction (MAE)

A) Without homogenization step

In the experiment the microwave CEM Corporation US MARS Xpress 5240 was used. To Teflon vessel with a volume of 50 mL 2.5 g of *S. limacinum* biomass as a beige powder was put and then 48 ml of hexane was added. The mixture was placed in a microwave oven; the magnetron power was set at 400 W and subjected to extraction for two hours. After the extraction the solvent

was removed on the rotary evaporator. This extract was stored at 4°C for analysis.

B) With homogenization step

10 grams of the raw material was transferred to a falcon tube and 20 mL of hexane was added. The solution was homogenized (ultrasonic homogenizer, 26 kHz, 200 W, Hielscher UP200HT) for 1 h. During the homogenization system was cooled on ice. The resulting mixture was placed in an ultrasonic bath (Cole-Parmer 8891) and sonicated for 30 minutes. The hexane was evaporated. The extraction of *S. limacinum* biomass after homogenized process was carried technique microwave assisted extraction. 2.5 g of *S. limacinum* biomass as a beige powder was put and then 48 ml of hexane was added. The mixture was placed in a microwave oven; the magnetron power was set at 400 W and subjected to extraction for two hours. After the extraction the solvent was removed on the rotary evaporator. This extract was stored at 4°C for analysis.

The ultrasound-assisted extraction (UAE)

A) Without homogenization step

10 g *S. limacinum* biomass was put into an Erlenmeyer flask, and then 200 mL of hexane was added. The mixture was placed in an ultrasonic bath (POLSONIC) and sonicated for two hours with suitably programmed intervals. After the extraction the solvent was removed on the rotary evaporator. This extract was stored at 4°C for analysis.

B) With homogenization step

10 grams of the raw material was transferred to a falcon tube and 20 mL of hexane was added. The solution was homogenized (ultrasonic homogenizer, 26 kHz, 200 W, Hielscher UP200HT) for 1 h. During the homogenization system was cooled on ice. The resulting mixture was placed in an ultrasonic bath (Cole-Parmer 8891) and sonicated for 30 minutes. The hexane was evaporated. The extraction of *S. limacinum* biomass after homogenized process was carried technique ultrasound-assisted extraction. 10 g *S. limacinum* biomass was put into an Erlenmeyer flask, and then 200 mL of hexane was added. The mixture was placed in an ultrasonic bath (POLSONIC) and sonicated for two hours with suitably programmed intervals. After the extraction the solvent was removed on the rotary evaporator. This extract was stored at 4°C for analysis.

3. THE EXTRACT ANALYSIS

The sample (approx. 5 mg) was mixed with 0.5 ml of tert-butyl methyl ether, 0.25 mL of a solution of trimethylsulfoxonium hydroxide in methanol (0.25 M), and 25 uL of internal standard (undecanoate methyl in tert-butyl methyl ether (Aldrich) 41.2 mg in 10 mL). Samples were injected after 1 minute of derivatization. Analysis of the extracts after derivatization was performed using a gas chromatograph Varian 450-GC. For injection 1 mL of sample was prepared as described in the following part of the procedure. The measurements were performed with the following conditions: injector temperature: 250°C; split

1:50; carrier gas: He, flow mL/min; Column: Agilent HP-INNOWAX, 30 m x 0.53 mm, film thickness: 1 micron; temperature program: 50°C isothermal for 2 min, linear gradient of 10°C/min to 240°C (20 min), 240°C isothermal for 22 min; Detector: FID; detector temperature: 250°C.

The evaluation of fatty acid methyl esters was carried out by comparing the retention times of the standards. The following patterns (as methyl ester) were used: butyric acid (C4: 0), valeric acid (C5:0), caproic (C6:0), caprylic acid (C8:0), pelargonic acid (C9:0), capric (C10:0), undecanoic (C11:0), lauric (C12:0), tridecanoic (C13:0), myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), margarinic (C17:0), stearic (C18:0), nonadecanoic (C19:0), arachidic (C20:0), heneicozaenoic (C21:0), behenic (C22:0), tricosanoic (C23:0), lignoceroic (C24:0), miristoleic (C14:1 n-5) cis-10-pentadecenoic (C15:1 n-5), palmitoleic (C16:1, n-7) cis-10-heptadecenoic (C17:1, n-7), oleic (C18:1, n-9), vaccenic (C18:1, n-7) petroselinic (C18:1, n-12) cis-11-eicosenoic (C20:1, n-9), erucic acid (C22:1, n-9), nervoic (C24:1, n-9), linoleic (C18:2 n-6), α-linolenic acid (C18:3 n-3), γ-linolenic acid (C18:3 n-6), stearidonic (C18:4 n-3), cis, cis-11.14-eicosadienoic (C20:2 n-6) cis, cis, cis-8,11,14-eicosatrienoic (C20:3, n-6) cis, cis, cis-11,14,17-eicosatrienoic (C20:3, n-3), arachidonic (C20:4, n-6); all-cis-5,8,11,14,17-eicosapentaenoic (C20:5 n-3), cis, cis-13,16-docosadienoic (C22:2 n-6), all-cis-7 10,13,16,19-docosapentaenoic (C22:5 n-3), all-cis-4,7,10,13,16,19-docosahexaenoic acid (C22:6, n-3).

4. RESULTS AND DISCUSSION

The *S. limacinum* in culture is in the form of spherical and their agglomerates (Figure 1). The compact spherical outer membrane is a significant barrier in the process of extraction solvent. Overcoming this barrier during extraction of fatty acids determined possibility of obtaining these components from the interior of the microorganism biomass. We developed a method of destruction of the outer membrane of *S. limacinum* by homogenization of system and were determine how this process influences on obtaining fatty acids by various methods of extraction. Following homogenization of biomass by the method described in Experiment is obtained completely free of spherical bodies (Figure 2).

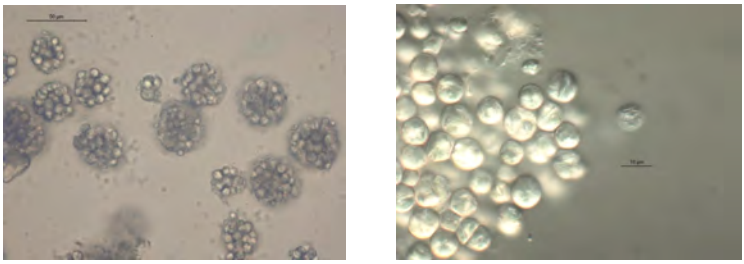

Fig. 1. The *Schizochytrium limacinum* biomass examined in light microscope (400x and 1000x magnification)

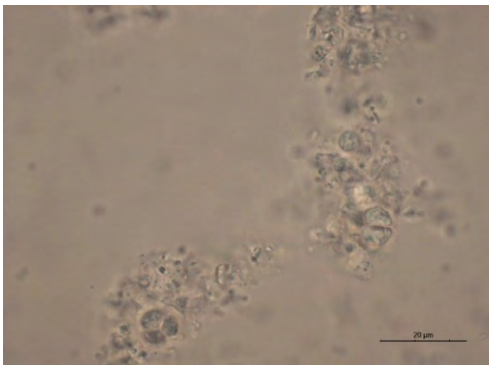

Fig. 2. The *Schizochytrium limacinum* biomass after homogenization step (400x magnification)

Figures 3–6 show the biomass after extraction process using different techniques. Spherical units or not fractured (Soxhlet extraction method; Figure 3) or retain your own structure being empty creations (SFE, MAE and UAE; Figures 4, 5 and 6 respective).

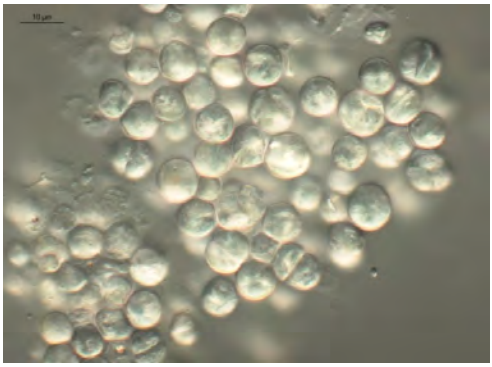

Fig. 3. The *Schizochytrium limacinum* biomass after Soxhlet extraction. Without homogenization step (1000x magnification)

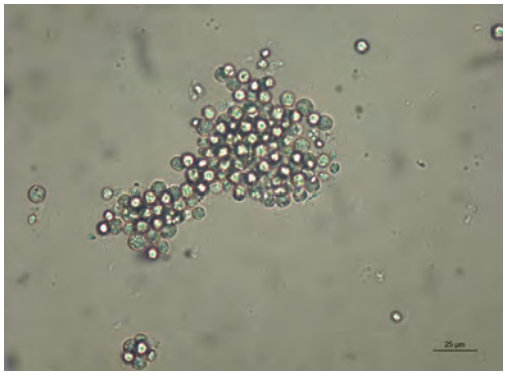

Fig. 4. The *Schizochytrium limacinum* biomass after supercritical fluid extraction. Without homogenization step (400x magnification)

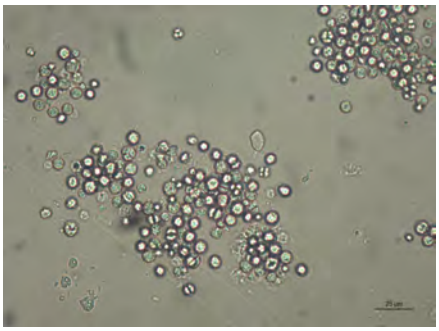

Fig. 5. ***S. limacinum*** **biomass after microwave assisted extraction. Without homogenization step (400x magnification)**

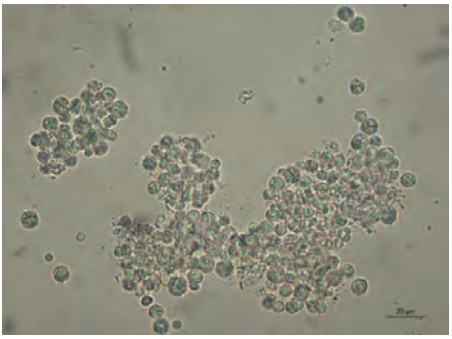

Fig. 6. ***S. limacinum*** **biomass after ultrasound assisted extraction. Without homogenization step (400x magnification)**

Based on the results of extraction efficiency for methods without the additional step of homogenization can be concluded that the most efficient method was SFE extraction (extraction yield is about 32.0%) compared to the other methods used (extraction yield for the Soxhlet extraction is 16.5%, for UAE is 17.0%, for MAE is 13.4%). The destruction of *S. limacinum* microorganisms using a homogenizer, in most cases, has a positive effect and resulting on increasing the efficiency of extraction. For all methods of extraction was observed significant extraction yield of fatty acids. The largest increase extraction yields observed in the case of technology Soxhlet extraction and microwave assisted extraction. For these two methods, the introduction of additional stages using ultrasounds resulted in an increase in yield about 30 percent (2.5 times). As in the case of a procedure without the use of a homogenization step, the highest extraction yield was observed in the SFE method (52.0%). For other methods, extraction yield amounted: 46.0% for MAE, 42.2% for Soxhlet extraction, 30.4% for UAE.

The eight extractions were carried out by four extraction techniques using either biomass or homogenized biomass under the conditions described in the experimental parts. The fatty acid composition in extract of *S. limacinum*

biomass were determined for all extracts. It was found that the fatty acid content in the extracts acquired by various techniques are similar. The method of extraction of the biomass without any pre-treatment or homogenization treatment does not affect the quality and quantity of fatty acid. Homogenization of biomass has only significant influence on the extraction yield.

It could be concluded that for obtaining fatty acids from *S. limacinum* biomass applying extraction process of biomass, the most efficient extraction methods are SFE and MAE with the using preliminary homogenization process of the material.

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THE INFLUENCE OF CERTAIN SUPERCRITICAL PLANT EXTRACTS ON BIOFILM FORMATION IN *PSEUDOMONAS AERUGINOSA*

Summary

In this study the antibacterial activity and the effect of supercritical extracts of hop (*Humulus lupulus* L.), industrial hemp (*Cannnabis sativa* L.) and lichen (*Usnea barbata* L.) on biofilm formation or disruption of the already formed biofilm in *P. aeruginosa* strains were assayed (PAO1, Br5, 50 and C14). The inhibitory effect of the extracts on biofilm formation was tested in 3 different concentrations that are below MIC values, or 1/2 MIC, 1/4 MIC and 1/8 MIC. For the research of biofilm disruption, the extracts were applied in concentrations two times stronger than MIC (2 x MIC). It was found that the applied supercritical extract of hop in concentrations of 6400, 3200 and 1600 μg/mL had expressed proliferative influence on PAO1 strain. The same extract at the concentration of 6.400 μg/mL showed the inhibitory activity on the production of biofilm in 50, Br5 and C14 strains which formed biofilms with reduced intensity (83%, 84% and 89%) compared to controls. Supercritical hop extract at concentrations of 3200 and 1600 μg/mL had inhibitory activity on the production of biofilm in Br5 and C14 strains which produced biofilm in 62–72% higher than their controls. Supercritical extract obtained from *Usnea barbata* showed a pronouncedly stimulating activity on the production of biofilm in all assayed *P. aeruginosa* strains. At concentrations of 12.800 and 6400 μg/mL, cannabidiol rich extract had a stimulating effect on all *P. aeruginosa* strains and an overproduction of biofilm occurred. The smallest concentration of the cannabidiol rich extract of 3200 μg/mL showed a surprisingly uniformed inhibitory effect on the production of biofilm in all 4 *P. aeruginosa* strains. The results of disruption of already formed biofilms during the time of exposure of biofilms for duration of 6 h showed that all of the extracts had a proliferative effect on formed biofilms. In contrast, extracts that were left to act on biofilms during 24 hours, in all cases showed degradation effect on the formed biofilm.

1. INTRODUCTION

1A. Scope of the research

Bacteria are considered to be the oldest living beings on the planet; they were formed 4 billion years ago. Thanks to their short generation time and the size of their populations, they are able to expand and grow faster than any other living creature. The ability to adapt to new environmental conditions in some significantly developed biological species is much smaller than in bacteria. Throughout evolution many biological species failed to adapt to some changes that had occurred suddenly and they disappeared from the face of the earth.

The ability of bacteria to rapidly evolve and adapt to new environmental conditions can best be seen through the problem of bacterial resistance to antibiotics. The phenomenon of bacterial resistance to antibiotics has taken the form of a real biological universal cataclysm in the recent years. The World Health Organization (WHO) has forecasted that until 2050 due to the ineffectiveness of antibiotics as a consequence of bacterial resistance, 300 million people worldwide will die prematurely [1]. The increase in the number of deaths due to infections by multidrug resistant strains of bacteria has come to the point where the scientific papers, and the media as well (newspapers, TV) are warning the humanity on the insolvability of the problem and the very obvious disastrous consequences which humanity is even now suffering from. Difficult to cure infections of people caused by multidrug resistant and panresistant strains of bacteria appear daily on all continents and in all countries which categorized the resistance as pandemic, which in scientific literature is compared to Spanish flu, AIDS and other infectious disease pandemics [2]. For the first time in history, from its founding, a session of the General As for sembly of the United Nations has been scheduled for September of 2016, on the topic of antibiotic resistance, and this problem will be treated as a forthcoming and very real threat to the survival of mankind.

The attempt by science and the mankind to “eliminate” bacteria as a type of living creatures that are marked as “undesirable” clearly resulted in a frightening response of these tiny organisms, which, in an unexpected historical twist, is now threatening to “eliminate” people.

In addition to the mechanisms of resistance to antibiotics, bacteria have many other mechanisms that for many years were unknown to science, and that allowed bacteria to survive undisturbed even in conditions where there was a real possibility of eradication (such as the start of the global use of antibiotics and disinfectants). In addition to the sensational discovery of the phenomenon of communication between bacteria (*quorum sensing*), one of these relatively recently discovered mechanisms is the ability of bacteria to form biofilms. Unlike the antibiotic resistance that occurs as a result of mutation, the ability to create biofilm is not considered evolutionary adjustment of the bacteria but their basic form of survival in the environment that exists as long as the bacteria

themselves, hundreds of millions of years [3]. Biofilm as a structure produced by the bacteria presents a physical barrier that protects the bacteria from a variety of external influences including antibiotics and disinfectants [4]. Certainly, not all bacteria have the same type of ability to form biofilms; some species have no ability to do this at all. Also, in some species of bacteria resistance to antibiotics is very rare, or it occurs in an insignificant percentage. *Pseudomonas aeruginosa* is not one of these species. In the last 10 years the problems of antibiotic resistance and the ability to form biofilms have met and merged with the same topicality and intensity in this particular type of bacteria, thus enormously increasing its importance in clinical practice [6, 7, 8]. Multidrug resistant strains of *P. aeruginosa* have emerged, which, besides being resistant to almost all antibiotics, form a biofilm, making infected people incurable patients [7, 8].

Because of all of the aforementioned, research on the antibacterial activity of non-antibiotic substances, including plant extracts, have been extremely topical for the past 10 years. In addition, scientists are searching for substances of synthetic or natural origin that have the ability to destroy already formed biofilms or the ability to inhibit the formation of biofilms [9].

In accordance with the aforementioned, in this study the antibacterial activity and the effect of supercritical extracts of hop (*Humulus lupulus* L.), industrial hemp (*Cannnabis sativa* L.) and lichen (*Usnea barbata* L.) on biofilm formation or destruction of the already formed biofilm in *P. aeruginosa* strains were assayed.

1B. Biofilm

Biofilm, or as it is also referred to as "a multicellular model of life organization in bacteria", is a relatively newly discovered phenomenon in microbiology. Biofilm presents a major challenge in modern science and medicine because it is a phenomenon that hinders the disinfecting process of surfaces and objects, and it also hinders successful treatment of bacterial infections in humans and animals with antibiotics [3, 4, 5, 9, 10]. It has been proven that biofilm is a natural form in which bacteria live in all ecological environments and that actually bacteria cannot survive in the external environment as unicellular or independent individuals (plankton form), but exclusively by uniting in the joint formation i.e. the biofilm structure [3, 4, 5, 9, 10].

In the simplest terms, biofilm is a formation that is generated by uniting bacteria attached to a surface that causes a secretion of extracellular polysaccharide matrix (EPS), which mainly consists of cellulose [3, 4, 5, 9, 10, 11, 12, 13, 14]. Thus, a thin layer (film) is formed on the contaminated surface within which bacteria live, multiply and excrete toxins unhindered. Biofilm cannot be removed by simply rinsing. Biofilms are extremely hard to prevent and eradicate, and modern strategies for combating pathogenic bacteria are insufficient when it comes to controlling pathogens organized in the biofilm. In

the biofilms the bacteria show increased resistance to stressful factors (draining, effects of antibiotics, disinfectants, heavy metal ions, UV radiation) [3, 4, 5, 9, 10, 11, 12, 13, 14]. Antibiotics and other antimicrobial agents have difficulty passing or do not pass at all through the biofilm matrix, which is called biofilm tolerance to antimicrobial agents. Not only are the microorganisms from biofilms protected from the operation of antimicrobials, they are also protected from the immunologic mechanisms of the infected individual.

The formation process of biofilms is extremely complex; it takes place in several stages and depends on a great number of factors related to the bacteria itself and their genome, as well as the environmental conditions. In addition to biofilms being formed in living infected tissues, the formation of biofilms occurs in inanimate environments contaminated with microorganisms. In previous tests, biofilms were found in pipelines, and not just sewage but also in the clean (drinking) water supply pipe systems, on urinary catheters used in human and veterinary medicine, on human and animal teeth, contact lenses, breast implants, prosthetic heart valves, dental implants, on the roots of various plants and in lung tissue of people who suffer from cystic fibrosis [3, 4, 5, 9, 10, 12, 13, 14]. Biofilms have occasionally been found in other biotic and abiotic environments, depending on numerous factors. It was found that the biofilms that formed in infected tissues (in the mucous membranes) are the reason for occurrence of serious chronic infections such as chronic bladder infection, chronic inflammation of the middle ear and sinus infections, chronic inflammation of the prostate, and persistent purulent skin infections (wounds, bedsores) caused by *E.coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The characteristic of these persistent infections is that they last for months or even years despite the disciplined implementation of antibiotic treatment with antibiotics that pathogens are sensitive to.

The complexity of biofilm, the mode of its formation, its structure and maintenance, are still a relatively big secret in science. Given that antibiotics provide little or no results in the prevention and treatment of infected tissues in which bacterial biofilms are formed, new non-antibiotic substances that might have an effect on bacterial biofilms are being researched. On the other hand, testing of new materials is being performed, materials to which bacteria cannot adhere, and the adherence of bacteria to the surface is the first stage and a prerequisite to the formation of biofilms [9].

1C. *Pseudomonas aeruginosa* and its importance in clinical medicine

Pseudomonas aeruginosa is a Gram-negative rod. It is covered with flagella, has an adhesive pili, endotoxin (lipopolysaccharide) and a capsule. Most strains have the ability to produce biofilm [6, 8, 9]. It also secretes exotoxin that interferes with protein synthesis in infected cells. *P. aeruginosa* produces 4 types of pigment and and these are as follows: *pyocyanin* (blue-green), *pyoverdin* (green-yellow), *pyorubin* (red) and *pyomelanin* (brown-black) (Figures 1 and 2).

Pseudomonas species are present almost everywhere: on the skin and mucous membranes, in all types of domestic and wild animals as well as birds, in soil, on plants, in current and stagnant waters, wells, walls and floors of rooms, egg shells, the skin of freshwater fish, etc. It is extremely resistant to external environmental conditions and is able to reproduce at temperatures of 4 to 42°C. This species can be isolated from virtually all types of materials and from all ecological niches. It has typical pigmented colonies with metallic glow (Figure 3) and specific smell (rotten fruits, lime).

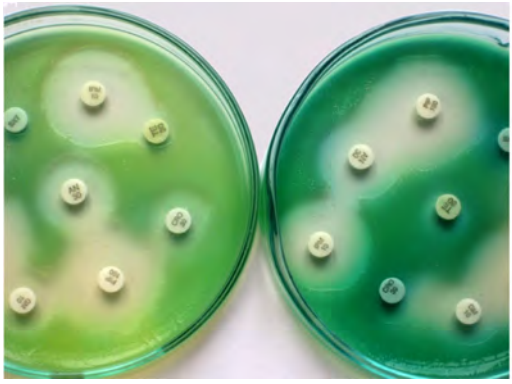

Fig. 1. (left) *P. aeruginosa* strains with different shades of pyocyanin

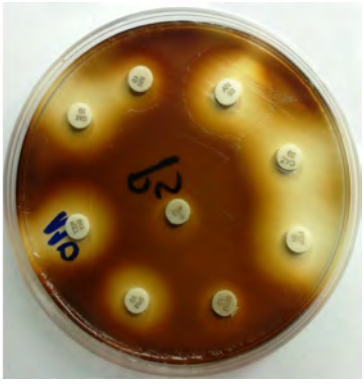

Fig. 2. (right) *P. aeruginosa* strain with pyomelanin

Fig. 3. *P. aeruginosa* colonies on Columbia blood agar – typical metallic glow is visible

In the food and beverage industry, *P. aeruginosa* plays an important role as a contaminant that is normally found in raw materials. Due to its ability to produce biofilm, but also because of its resistance to the large number of disinfectants, it can survive for years on the inside walls of water tanks, dishes, machinery and other industrial elements in food factories, preventing the production of microbiologically safe foods [15]. It is necessary to emphasize that

P. aeruginosa has no importance from the food safety standpoint because it does not fall into the category of foodborne pathogens but it does affect the rapid breakdown of food. It is particularly often found in fish and fish products [6, 15].

Regarding the resistance of these species, there is data on strains that were found multiplying undisturbed in concentrated solutions of some disinfectants [16]. There are known cases in the world, where the entire hospital wings, in which the *P. aeruginosa* species should not be present (intensive care units and maternity wards), had to be temporarily closed due to inability to remove *P. aeruginosa* from the walls, floors and other surfaces by using disinfectants [17, 18]. In veterinary clinics *P. aeruginosa* may end up staying on the anesthesia equipment or in the infirmary in places with increased humidity [12, 13]. *Pseudomonas* species are susceptible to desiccation, and in dry environments they quickly deteriorate. Whenever possible, the objects and surfaces should be exposed to direct sunlight [6].

Pseudomonas aeruginosa rarely cause infection in healthy individuals. Infection by this pathogen happens almost in all cases where there is an immunocompromised status, stress, illness, surgery or vitamin deficiency [7, 8, 9]. In human medicine the above mentioned species have significance as ‘as it is’ the statistically most common cause of serious in-hospital (nosocomial) infections in immunocompromised and surgical patients as well as burn patients [3, 7, 8, 9]. In outpatient settings, *P. aeruginosa* causes complicated infections in people with pre-existing diagnoses (diabetes, cystic fibrosis, AIDS, leukemia, cancer, patients with catheters, patients on immunosuppressive therapy) or in the elderly (bedsores). In the above mentioned groups of patients, *P. aeruginosa* causes a wide range of infections, from the inflammation of the bladder to wound infections, conjunctiva of ears, and even pneumonia and sepsis. The exception is healthy people that often engage in swimming and who, because of the constant moisture in the ears, are predisposed to frequent infections of the outer ear canal caused by *P. aeruginosa* species that are normally found in pool water (so-called "swimmer's ear").

Pseudomonas aeruginosa is intrinsically resistant, or it may be better to say naturally insensitive to most antibiotics, including ampicillin, amoxicillin with clavulanic acid, Cephalosporins of the I and II generation type and most III generation cephalosporins, tetracycline, chloramphenicol, sulfamethoxazole with trimethoprim, neomycin, streptomycin, kanalycin, and other [6, 7, 8, 19, 20]. Therefore, the choice of drugs for the treatment of infections caused by this species is significantly limited and it can be a major problem in clinical practice. Therefore, for the treatment of pseudomonas infections, exclusively some of the so-called anti-pseudomonal antibiotics are used that are in most cases intended for intravenous use.

However, the problem of antibiotic resistance of *Pseudomonas* species does not end at this point. Similar to other types of bacteria, *Pseudomonas* species are capable of horizontal resistance gene transfer or through mutations they become resistant to the anti-pseudomonal antibiotics [7, 8, 19, 20]. In this regard, the

infections caused by *P. aeruginosa* species are not harmless at all from the therapy standpoint due to severe multidrug resistance to a considerable number, or even all available antibiotics.

1D. Scientific data on inhibition of biofilm production with plant extracts

Scientific literature has very little data on the inhibition of biofilm formation in bacteria by using plant extracts; and almost no data at all on the supercritical extracts and their application in creating biofilm inhibition.

There is scarce data available on the activity of some synthetic cannabinoids (HU-210) on the formation of biofilm in *Vibrio harveyi* [21]. It was established that HU-210 inhibits *quorum sensing* in *Vibrio harveyi* population resulting in inhibition of biofilm formation. However obtained results were uneven; only 1 strain was inhibited from producing the biofilm and other strains showed no inhibition with HU-210.

In a very interesting study by Różalski et al, it was found that supercritical hop extract as well as a bioactive substance that is obtained through a process of supercritical fluid extraction, called xanthohumol, have relatively strong inhibitory effect on formation of biofilms in *S. aureus* species [22]. It was found that hop extract and xanthohumolum in subinhibitory concentrations of 1/2 MIC have extremely strong inhibitory effect on the formation of biofilm, which was reduced by 80%. Same substances also have significant inhibitory effect in concentrations of 1/4 and 1/8 MIC. However, it is necessary to emphasize that plant extracts in general have demonstrated better antibacterial activity against Gram-positive bacteria, such as *S. aureus* [23, 24]. Strong antibacterial activity of the hop extract and xanthohumolum on *Staphylococcus aureus* and other staphylococci has already been proven several times and MIC values of these bioactive substances in the staphylococci can be compared with the MIC values of antibiotics [24].

Bjamsholt at al., in their studies have proven that crude garlic extract has strong inhibitory effect on the quorum sensing system of *P. aeruginosa* which results in cessation of production of biofilm in these species [25]. This extract had no direct inhibitory activity on *P. aeruginosa*, instead it repressively affected the function of about 167 genes from quorum sensing system in this type of bacteria. The authors also conducted their experiment on laboratory mice in '*in vivo*' conditions proving that the administration of crude garlic extract can lead to healing of chronic pulmonary infection caused by *P. aeruginosa* in lung cystic fibrosis.

A methanolic extract of the plant *Euphorbia hirta* L. in low concentrations of 0.5–1 mg/ml showed inhibitory effect on formation of biofilm in *P. aeruginosa* strains, whereby it was discovered that this extract prevents adhesion of the strains on to the polystyrene plate [26].

In a scientific paper of Han-Shin Kim, it was proven that toluene ginger extract in concentrations of 1–10% inhibits the formation of biofilm in *P. aeruginosa* strains by 39–56% [27].

Kerekes et al., while looking at the problem arising from bacterial biofilms in the food industry, states that sage, thyme, oregano, and cinnamon essential oils were highly effective in inhibiting the formation of biofilms in the *L. monocytogenes* and *S. aureus* species [28].

Given that most pathogenic species of bacteria have the ability to produce biofilm, this area of research is extremely large and the possibilities are enormous, and it's certain that this area of science is still relatively new and young.

2. MAIN PART

2A. Materials and methods

Strains used in experiments

Biofilm quantification assays and biofilm disruption assays were performed with 4 strains of *Pseudomonas aeruginosa* (Table 1).

Table 1. *Pseudomonas aeruginosa* strains used in this investigation

STRAIN	ORIGIN
PAO1	Laboratory strain
50	Isolated from eye infection (Dog)
Br5	Isolated from wound infection (Human)
C14	Isolated from fish

All investigated strains were previously isolated using conventional microbiological methods from the specimen routinely delivered to Department for Microbiology, Faculty of Veterinary Medicine, Belgrade. The exception was the *P. aeruginosa* strain marked as PAO1 NCTC10332 which is defined as a laboratory strain used as a referential control strain for biofilm production [29].

Plant extracts used in experiments

Antibacterial and antibiofilm activity of supercritical extracts of hop (*Humulus lupulus* L.), industrial cannabis (*Cannabis sativa* L.), and lichen (*Usnea barbata* L.) was tested. Commercially available supercritical hop extract was obtained from the INS, Poland (*New chemical syntheses Institute*, Pulawy), commercially available lichen supercritical extract (containing usnic acid > 90%) was purchased from Flavex Germany and Cannabidiol rich extract (60% of cannabidiol) of industrial cannabis, which was prepared for the experimental part of this research, was purchased from BAFA GmbH.

Research on antibacterial activity of supercritical plant extracts

The main precondition for testing the inhibition of biofilms in bacteria and the destruction of already formed biofilm, is determining the MIC value (minimal inhibitory concentration) of the substance to be tested. For the determination of MIC values of plant extracts, broth microdilution method was used in accordance with the CLSI prescription [30]. Cation adjusted Mueller Hinton II broth was used as a prescribed culture medium (CAMHB, Becton Dickinson). Dimethyl sulfoxide (DMSO, Serva) was used as the solvent for the herbal extracts. The only modification of the method was the usage of plant extracts instead of antibiotics. The investigated concentrations of plant extracts were 25.600, 12.800, 6400, 3200, 1600, 800, 400, 200, 100, 50, 25 and 12.5 expressed in μg/mL. The extracts were dissolved in DMSO at 256 mg/mL, then 1:10 dilution with CAMHB was made. Titration was performed in microplate wells as previously described [24, 30]. The final bacterial innoculum density of 5×10^5 CFU/mL was achieved by adding 5 μl of $1–2\times10^7$ CFU/mL suspension of investigated strain in microplate wells with 100 μl of previously added CAMHB. Microplates were incubated for 18–24 h at 37°C. MIC values were determined as the lowest concentration of an antimicrobial agent that prevents visible growth of microorganism.

Inhibition of biofilm formation and biofilm disruption assays

For the research of biofilm inhibition, the extracts were applied in concentrations less than the MIC values (Table 2). More precisely, the inhibitory effect of the extracts on biofilm formation was tested in 3 different concentrations that are below MIC values, or 1/2MIC, 1/4 MIC, and 1/8 MIC. For the hop supercritical extracts assay, investigated concentrations were 6400, 3200 and 1600 μg/mL, and lichen and cannabidiol rich supercritical extracts were used in concentrations of 12.800, 6400 and 3200 μg/mL.

For the research of biofilm disruption, the extracts were applied in concentrations two times stronger then MIC (2 x MIC) (table 3). The hop supercritical extract was used in concentration of 25.600 μg/mL, and lichen and cannabidiol rich supercritical extracts were used in concentrations of 51.200 μg/mL.

Table 2. Final concentrations of supercritical extracts used in inhibition of biofilm formation assays

Extract	Final concetration of extracts used in inhibition of biofilm formation assays Concentration lower than MIC (μg/mL)		
Humulus lupulus SFE (MIC 12.800 μg/mL)	6400	3200	1600
Canabidiol rich *Cannabis sativa* SFE (MIC 25.600 μg/mL)	12.800	6400	3200
Usnea barbata SFE (MIC 25.600 μg/mL)	12.800	6400	3200

Table 3. Final concentrations of supercritical extracts used in disruption of biofilm assays

Extract	Final concentration of extracts in biofilm disruption assays Concentration higher than MIC (μg/mL)
Humulus lupulus SFE (MIC 12.800 μg/mL)	25.600
Canabidiol rich *Cannabis sativa* SFE (MIC 25.600 μg/mL)	51.200
Usnea barbata SFE (MIC 25.600 μg/mL)	51.200

Inhibition of biofilm formation assays were performed in microtiter plates (Sarstedt 96 microtiter plates with flat bottom) using a crystal violet staining of adherent cells [11, 34]. Overnight cultures of *P. aeruginosa* strains were subcultured (OD_{600} = 0.2) at 37°C in LB (Luria-Bertani) liquid medium (Tryptone 10 g (Oxoid, UK), NaCl 10 g (Oxoid, UK), Yeast extract 5 g (Oxoid, UK) per Liter) with and without investigated extracts in 96-well microtiter plates. After 24 h growth, free (detached) cells were removed, wells were washed with PBS (Phosphate saline buffer, Dulbeco's, Serva) and biofilms fixed with 100 μL of 99% (v/v) methanol (Serva), followed by staining with 0.4% (v/v) crystal violet (Merck). After washing, crystal violet was solubilized with 150 μL of glacial acetic acid (33%, v/v, Merck) and the absorbance was measured at 540 nm on microtiterplate reader (Infinite 200 PRO, TECAN). The biofilm formation assay was performed in six wells and repeated twice. Relative percentages were calculated upon comparing with the negative solvent vehicle control (DMSO).

Formula: (A-D-B)/(C-D) x 100 where:

A – Optical density measured after the activity of plant SFE extract on biofilm formation
B – Optical density of plant SFE extract in LB medium
C – Optical density of investigated strain with DMSO
D – Optical density of sterile LB medium

Values presented are mean values of four replicates of two independent experiments.

Biofilm disruption assays were performed in 24-well microtiter plate and 96-well plate as previously described [11, 34].

Biofilm disruption assay in 24-well microtiter plate (Sarstedt 24 microtiter plates with flat bottom) was performed with *P. aeruginosa* cultures grown overnight in M9 medium at 37°C with shaking at 180 rpm and subcultured (OD_{600} = 0.01) in 24-well microtiter plate (1% v/v inoculums in 1 mL M9 medium supplemented with 20 mM glucose) and further incubated at 37°C with 180 rpm shaking. Investigated extracts were added at the concentration of 12.800 μg/mL (Hop SFE), 25.600 μg/mL (lichen SFE), 25.600 μg/mL

(Cannabidiol rich SFE) after 6 h of growth. After 1 h of exposition, free (detached) cells were removed, wells washed with PBS and adherent cells were stained with 0.4% (v/v) crystal violet.

Biofilm disruption assay in 96-well microtiter plate (Sarstedt 96 microtiter plates with flat bottom) was performed with overnight cultures of *P. aeruginosa* strains subcultured (OD_{600} = 0.2) in LB liquid medium and grown in 96-well microtiter plate without investigated extracts for 24h. After 24 h, medium was removed and wells were washed twice with PBS. Fresh LB liquid medium with investigated extracts in concentrations: 12.800 μg/mL (hop SFE), 25.600 μg/mL (lichen SFE), 25.600 μg/mL (Cannabidiol rich SFE) were added in each well. After incubation (exposition) of further 24 h, microtiter plates were washed and adherent cells were stained with 0.4% (v/v) crystal violet as previously described for biofilm quantification assay.

Controls

As controls, the DMSO solutions were placed in LB mediums in the same concentrations in which DMSO is found in the extract solutions (1 ml of DMSO) dissolved in 9 mL of LB medium which yielded a starting, highest concentration of DMSO, and then its double dilution was carried out in microtiter plate wells.

Also, controls of the extracts were set up in LB medium in all of the aforementioned concentrations, without the inoculated bacteria.

Controls of biofilm production for all 4 assayed *P. aeruginosa* strains were obtained after the strains were cultured in optimal conditions without the presence of inhibitory factors, and obtained results were taken as 100% of biofilm production. After exposing of the investigated strains to activity of supercritical extracts, results were obtained that were presented as a mean percentage value of the amount of formed biofilm in comparison with 100% of the control biofilm. The quantity was either lower than the control, which was considered as inhibition of biofilm formation, or higher than the control, which was considered to be the increased proliferation of biofilm, or its overproduction.

2B. Results

Results of antibacterial activity of supercritical extracts on the P. aeruginosa strains.

In this part of the investigations, MIC values were determined of all assayed extracts on the *P. aeruginosa* strains. Relatively uniformed results were obtained in all tested strains. Supercritical hop extract for all tested *Pseudomonas aeruginosa* strains had MIC values of 12.800 μg/mL. Cannabidiol rich supercritical extract and supercritical *Usnea barbata* extracts had identical MIC values for all assayed *P. aeruginosa* strains – 25.600 μg/mL.

Results of inhibition of biofilm formation with supercritical extract of hop

While testing the activity of supercritical hop extract, uneven results were obtained. It was found that the applied extract in concentrations of 6400, 3200 and 1600μg/mL had expressed proliferative influence on PAO1 strain. The treated strain in the presence of all concentrations of hop SFE increased the production of biofilm or proliferated, so the obtained values of biofilm formed, were around 200% as compared to controls.

Supercritical hop extract at a concentration of 6.400 μg/mL showed the inhibitory activity on the production of biofilm of *P. aeruginosa* strains as strains marked as 50, Br5 and C14 formed biofilm with reduced intensity (83%, 84% and 89%) compared to controls.

Supercritical hop extract at concentrations of 3200 and 1600 μg/mL did not show inhibitory activity but no stimulating effect either on the production of biofilm formation in *P. aeruginosa* strain 50 because this strain, in the presence of the extract, formed biofilm of almost the same intensity as the control (98% and 99.1%). Supercritical hop extract at concentrations of 3200 and 1600 μg/mL had inhibitory activity on the production of biofilm in *P. aeruginosa* strains Br5 and C14 because these strains produced biofilm in a smaller percentage than their controls (62–72%). In none of the presented cases did supercritical hop extract have completely inhibitory activity on the production of biofilm in *P. aeruginosa* strains. The results of this part of the research were presented in Figure 4.

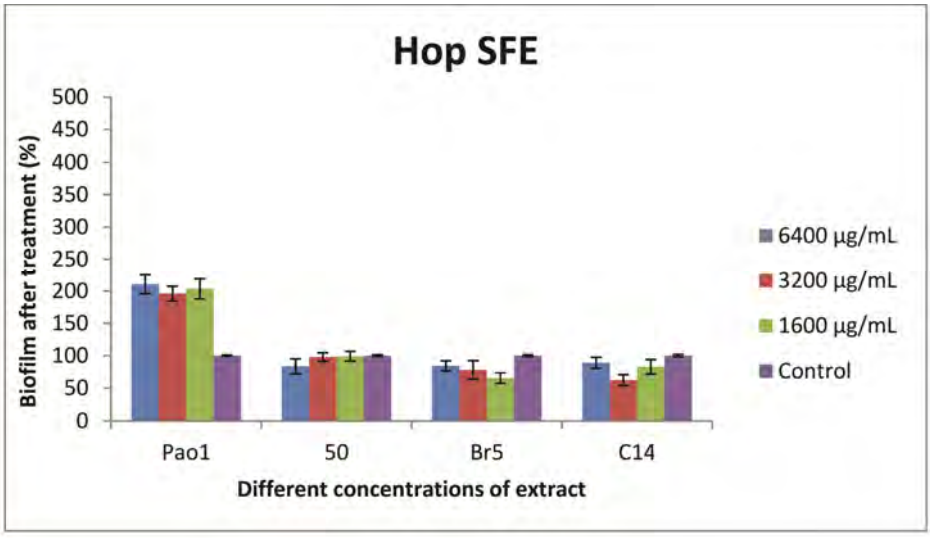

Fig. 4. Influence of hop SFE (supercritical extract of *Humulus lupulus* L.) on biofilm formation of *P. aeruginosa* strains

Results of inhibition of biofilm formation with supercritical extract of lichen Usnea barbata

Supercritical extract obtained from *Usnea barbata* showed a pronouncedly stimulating activity on the production of biofilm in all assayed *P. aeruginosa* strains. The expressed overproduction of biofilms occurred in all strains cultured in medium with lichen extract at a concentration of 12.800 μg/mL. At this concentration, the *P. aeruginosa* PAO1, Br5 and C14 strains have produced approximately 300% of biofilm compared to the control, and the 50 strain nearly 500%. The only exception happened in the case of the Br5-Usnea barbata extract strain at a concentration of 3200 μg/mL which showed an inhibitory effect on the 50 strain, since this strain at the stated concentration produced around 60% of the biofilm as compared to its control. The results of this part of the investigation are shown in Figure 5.

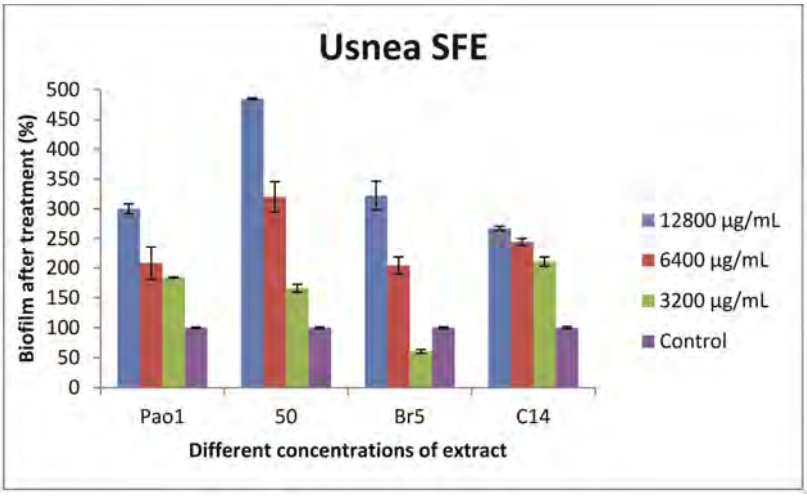

Fig. 5. Influence of lichen SFE (supercritical extract of *Usneabarbata*) on biofilm formation of *P. aeruginosa* strains

Results of inhibition of biofilm formation with cannabidiol rich supercritical extract of Cannabis sativa

The most interesting results were obtained in researching the influence of Cannabidiol rich extract obtained from industrial *Cannabis sativa*. At concentrations of 12.800 and 6400 μg/mL, cannabidiol extract had a stimulating effect on all *P. aeruginosa* strains and an overproduction of biofilm occurred. The smallest concentration of the extract of 3200 μg/mL showed a surprisingly uniformed inhibitory effect on the production of biofilm in all 4 *P. aeruginosa* strains (PAO, 50, Br5 and C14). The PAO, Br5 and 50 strains produced 70–80% of biofilm as compared to controls. The strongest inhibitory effect cannabidiol rich extract showed at a concentration of 3200 μg/mL on the *P. aeruginosa* C14 strain that produced only 33% of the biofilm as compared to its control. The results of this part of the investigation are shown in Figure 6.

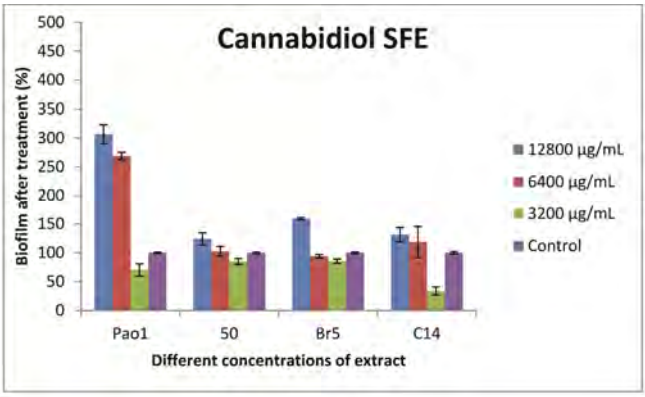

Fig. 6. Influence of cannabidiol rich SFE (supercritical extract of *Cannabis sativa* L.) on biofilm formation of *P. aeruginosa* strains

Test results of disruption of already formed biofilms

The effects of the assayed extracts on biofilms were tested during the time of exposure of biofilms for the duration of 6 h (in 24 wells microtiter plates) and 24 h (in 96 wells microtiter plates).

After 6 h of activity, all of the extracts showed a proliferative effect on formed biofilms. In other words, all biofilms continued to proliferate so that the OD readings and the calculation of obtained values compared to controls showed values of over 100%, which was the control value. Hop extract had the weakest proliferative effect and biofilms of *P. aeruginosa* strains further proliferated under the influence of the hop extract to about 300%, compared to the controls. Cannabidiol rich extract also had strong proliferative effect on formed biofilm which further proliferated to about 400–900%, compared to the controls. *Usnea barbata* extract had the strongest proliferative effect, under the influence of which, *P. aeruginosa* biofilms proliferated to a value 10 times higher than the control. The results of this part of the investigation are shown in Figure 7.

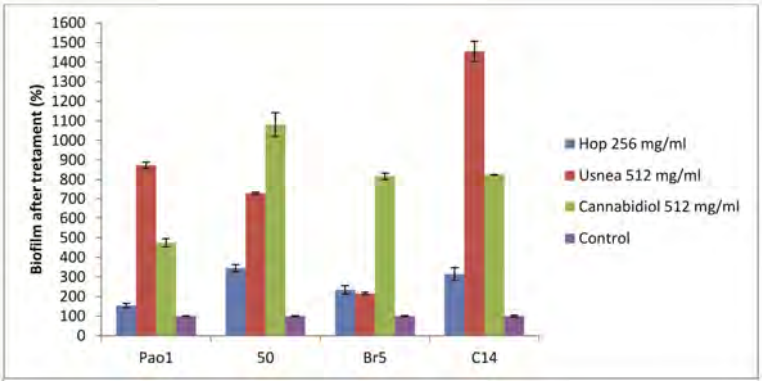

Fig. 7. Influence of supercritical extracts of hop, hemp and lichen on disruption of already formed biofilms of *P. aeruginosa* strains after 6 h of exposure

In contrast, extracts that were left to act on biofilms formed during the 24 hours, in all cases showed degradation effect on the formed biofilm. In this section results were presented as the mean value of degraded biofilms of all assayed *P. aeruginosa* strains (Figure 8). After 24 h of activity, supercritical hop extract degraded the treated biofilms by 48.26% on average. Supercritical lichen extract degraded all formed biofilms by 63.28% on average, and cannabidiol rich extract degraded all biofilms by 42.18% on average.

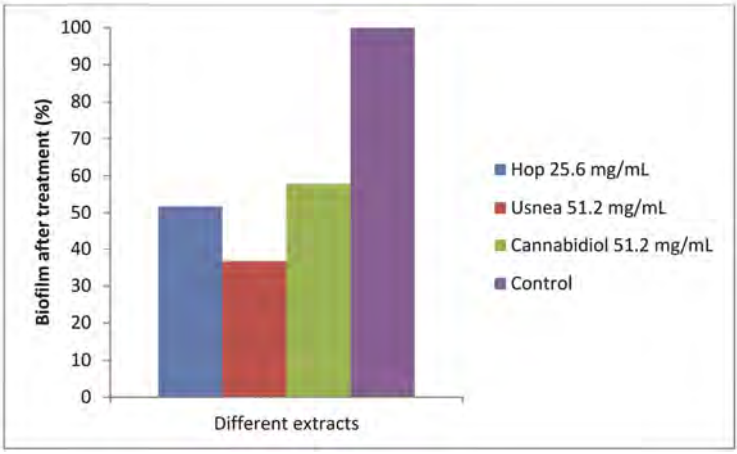

Fig. 8. Influence of supercritical extracts of hop, hemp and lichen on disruption of already formed biofilms of *P. aeruginosa* strains after 24 h of exposure

2C. Discussion

In literature, it is evident that assays for the inhibition of bacterial biofilms are carried out in two ways. One way is to use certain substances to try to stop communication between bacteria, i.e. to try and interfere with the phenomenon known as *quorum sensing*. This phenomenon was discovered relatively recently and is still the subject of extensive and intense studies; and it is still considered as sort of a secret to science. It has been observed that bacteria communicate with each other by secreting i.e. producing signaling proteins that some scientists even call hormones [35, 36, 37, 38]. Signaling proteins are secreted when a certain stimulus is affecting the bacteria, and through synthesized signaling protein bacteria "inform" other bacteria of the present stimulus. Based on this information, other bacteria begin a certain activity in accordance with the present stimulus although not all bacteria have come in contact with these stimuli yet. The stimuli can be positive or negative for bacteria, and bacteria take action in accordance with the fact if they are in certain danger or not (presence of antibiotics, presence of leukocytes, presence of food, etc.). Biofilm formation is highly dependent on the phenomenon of *quorum sensing* [39, 40, 41]. Actually, it is a very important moment in which bacterial populations begin the formation of biofilm, or a moment in which all the bacteria begin synchronized

secretion of extracellular matrix, since synchronized, simultaneous secretion of extracellular matrix is the main prerequisite for successful formation of biofilms. Thus testing of inhibitory effects on the synthesis of biofilm are based on the analysis and the chemical characterization of signaling proteins important for the initiation of synthesis of biofilm and the possibility that these signaling proteins can be neutralized or their excretion can be prevented in some way [42]. This research is very complex, lengthy and expensive and are only emerging. It should be noted that *quorum sensing* is an extremely complex phenomenon, which includes the activity of hundreds of genes and therefore signaling proteins as well.

Another way to test formation inhibition of bacterial biofilms is the one that was used for this paper. Bacterial strain that demonstrated formation of biofilms is selected and this strain is subjected to certain substances that are suspected to have inhibitory effect on biofilm formation. The most common method of testing biofilms is testing that is performed on polystyrene plates where the possibility of adhesion of bacteria to the surface of polystyrene in certain mediums is actually examined, this being the basic precondition for the initiation of the formation of biofilms. We should not forget that the ability to form biofilms in bacteria is categorized in virulence factors, i.e. bacteria that are capable of forming biofilms also have greater ability to cause a disease [3, 42].

However, in this second category of assays, it was observed that many substances that clearly and unambiguously show strong antibacterial activity in subinhibitory concentrations, actually encourage the formation of biofilms. The results of the research was published, which allowed to establish that some *Acinetobacter baumannii* strains in the presence of subinhibitory concentrations of imipenem (which is usually the antibiotic of choice for treating an infection caused by *Acinetobacter* species) produce 3 times more biofilm than when imipenem was not present [43]. Similarly, it was found that some *P. aeruginosa* strains in the presence of subinhibitory concentration of aminoglycosides, particularly tobramycin, form biofilm increasily [44]. More precisely, the subinhibitory concentration of this antibiotic stimulate the formation of biofilms in some *P. aeruginosa* strains. Again, this is not a rule; only some strains of bacteria behave this way, while this phenomenon does not occur in other strains. Also, differences occur in the type of assayed substances. For example, the presence of subinhibitory concentrations of tetracycline in *Acinetobacter baumannii* strains does not cause inhibition nor does it stimulate the formation of biofilms [43].

Similarly, in this study, we got very interesting results, which showed that the extracts that have antibacterial activity, and which in certain concentrations (MIC) clearly inhibit the growth and multiplication, i.e. kill assayed strains of bacteria, in subinhibitory concentrations, have extremely strong stimulating effect on the formation of biofilms in *P. aeruginosa* strains.

Why it is so, is still unknown. In several scientific papers there is a potential explanation. And that is that the presence of a substance that has antibacterial

activity and that can potentially kill bacteria, has a very stressful effect on the treated strains [3, 43, 44]. Bacteria recognize the potential "killer". This is the "trigger" for their enhanced and coordinated activity in the production of biofilm which has a protective function as it should hinder or completely prevent penetration of present antibacterial substance (in this case a plant extract) through biofilm.

Another potential explanation could also be the chemical composition of the applied extracts. For example, the reference medium for testing the formation of biofilms in bacteria is LB broth. If, when glucose is added to the LB broth, biofilm formation is better, the glucose is a stimulus [11, 34, 43]. In *P. aeruginosa* strains it was noted that the strongest stimulus for biofilm formation is elemental iron [45]. During the experiments it was found that the removal of iron from the medium in which *P. aeruginosa* strains were being cultivated, leads to the cessation of biofilm production (not only this, but some other virulence factors are lost as well). On the other hand, the addition of iron to the medium acts as a strong stimulus for biofilm production. Therefore, it is known that plant extracts obtained through the process of supercritical fluid extraction have extremely complex chemical composition with more than hundreds of detected components. It is not excluded that the extracts, of hop and lichen in particular, have an additional nutritional stimulus on the present strains. The extracts probably contain vitamins, but also many other substances including iron, which can have a strong stimulating effect on the formation of biofilms.

This, however, does not explain the very strong inhibitory effect of cannabidiol rich extract applied in the minimum concentration of 3200 μg/mL on all assayed *P. aeruginosa* strains. This is therefore an area that requires additional studies in order to obtain precise results and explanations.

3. CONCLUSIONS

It is clear that the supercritical extracts of hop (*Humulus lupulus* L.), hemp (*Cannabis sativa* L.) and lichen (*Usnea barbata* L.) may have the potential application in clinical practice as antibacterial agents for the treatment of local skin infections caused by various bacteria species, including *P. aeruginosa*. However, it is important to note here that in most cases of possible application of these extracts it cannot be forgotten that their subinhibitory concentrations that are lower than the MIC value may have adverse consequences, because, according to our results, it can lead to the proliferation of biofilm which in turn can lead to the infections becoming persistent. This is true for all the applied concentrations of all extracts except for cannabidiol at a concentration of 3200 μg/mL, which inhibited the production of biofilms in all *P. aeruginosa* strains.

In any case, further research is needed to give a clearer insight into this issue. The question remains: which extract components lead to the proliferation of biofilms and through which mechanisms. By changing the parameters of supercritical fluid extraction it is possible to eliminate these components from the final extract, which in turn can enable wider application of extracts in this area.

Acknowledgments

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LUPULIN AS THE VALUABLE SOURCE OF BIOACTIVE COMPOUNDS

Summary

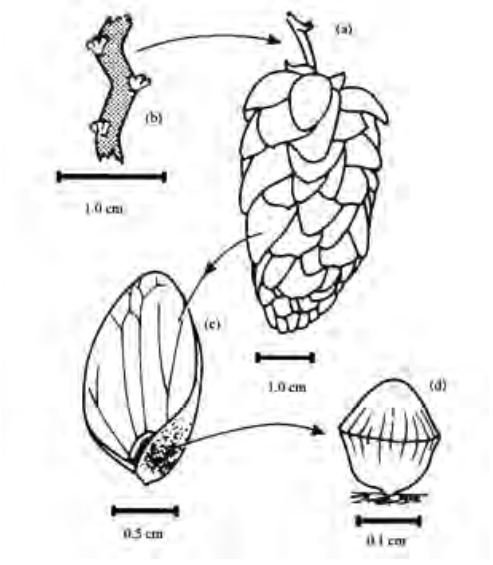
In the presented study the results of the analysis of the lupulin with the use of the carbon dioxide in a supercritical state has been provided. Moreover, this work studies also the influence of the parameters on the extraction efficiency and the quality of the obtained products, in which α-acids and β-acids were analyzed using liquid chromatography. In the researches, the methods of the experiment planning, referred to as Design of Experiment (DoE) as well as another method, called Response Surface Methodology (RSM) were used.

1. INTRODUCTION

Hops (*Humulus lupulus L.)* grow in their natural state in the humid thickets, forests but also at the fences and roadsides. They are found in the temperate zone of Europe (including Poland), Asia and North America. Hops are perennial dioecious plant belonging to a hemp family (Cannabinaceae), of which the female infructescences, referred to as strobilus are important material both in brewing and cosmetics industry. The mature hope cone contains the lupulin grains on the inside of the bracts and enveloping leaves. The lupulin grains are glandular secretions and are a major source of bitter and aromatic substances. Light yellow, shiny lupulin acorns of hops strobilus are placed at the base of the bracts, which include all the valuable material.

In the constructions of strobilus, there are distinguished:

- a columella in the form of a knee-shaped axis
- leaves and bracts, the number of which is from 40 to 100.



d)

Fig. 1. The hops strobilus schema and its parts: (a) strobilus, (b) a central core in the cone (c) bract, (d) lupulin [1]

Table 1. The chemical composition of hops according to Dylkowski [2]

Components	Content %
Bitter substances	18
Hop oils	0,5
Polyphenols	3,5
Protein	20
Minerals	8

Table 2. The chemical composition of hops according to Pazera [3]

Components	Content % in dry matter
Water	10-12
Total resin:	14-25
– α-acids	4,5-15
– β-acids	7-10
Hard resins	3-3,5
Hop oils	0,6-2,8
Polyphenols (tannins)	4,5-16
Monosaccharides	4,5-10
Proteins	13-24
Fatty acids	0,06-0,22
Minerals	8-12
Lipids and waxes	Traces to 3,4

Hops is the spice used not only for beer production. It is also used in the cosmetics industry and medicine.

In the cosmetics and treatment of the skin diseases strong antibacterial and antifungal properties of hops have long been used. Hops in enriched with phytohormones, which have beneficial effects on the metabolism of the skin and is therefore increasingly used in the products, referred to as anti-aging products.

In the brewing, dried hops are less frequently used. Nowadays, hops preparations, such as powders and hops granulates as well as isomerised hop extracts are common. Even if, hops may be added to beer in the small quantities, it plays an important role in the formation of taste and smell. The most important feature of hops is that it creates a characteristic bitterness [4].

Hops is made of over 1000 different chemical substances, the most important of which in terms of their properties are hops acids, such as α-acids and β-acids, but also essential oils and flavonoids (quercetin, kemferol, xanthohumol, rutin).

α-acids is a mixture of optically active homologous, slightly soluble in the water. The most leading are humulone and co- and adhumulone. The most important reaction that α-acids undergo from a technological point of view is the isomerization. During these changes, cyclohexane chemical structure of these compounds is converted to a cyclopentane structure, which results in obtaining iso- α-acids, which are dissolved. Bitter hops acids isomerization during the brewing in the brewing house give the beer a typical, pleasant bitter taste and are the main factors responsible for the stability of the foam. That's the reason why α-acids are under the great importance.

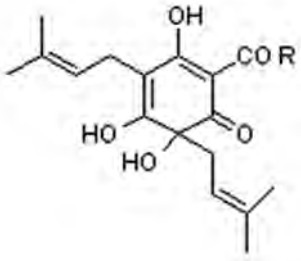


Fig. 2. α-acids (R-acyl side chain of the molecule)

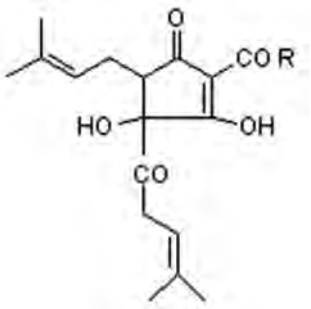
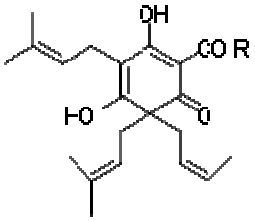


Fig. 3. iso-α-acids

β-acids are the second important component of the soft resins of hops. It is a mixture of lupulons, which are optically inactive hexacyclic substances. This group of compounds is poorly soluble in water, thus they cannot significantly affect the flavour of beer. However, they undergo oxidation processes to form hulupones, which are bitter and have the influence on the bitterness of some beers.



***Radicals (R):**

- $CH_2CH(CH_3)_2$ - isovaleric acid rest – HUMULONE, LUPULONE, ISOHUMULONE
- $CH(CH_3)_2$ - butyryl rest – COHUMULONE, COLUPULONE, ISOCOHUMULONE
- $CH(CH_3)CH_2CH_3$ - 2-methylbutyryl rest – ADHUMULONE, ADLUPULONE, ISOADHUMULONE

Fig. 4. β–acids

The other important components of hops are also essential oils, commonly referred to as hop oils. The total content of essential oils varies from 0.3 to 3.0 ml per 100 grams of hops. It is a complex mixture of essential substances, from which approximately 300 volatile components were distinguished. During the maturation of hops, essential oils are secreted to lupulin, passing strobilus a distinctive hop aroma. Different composition of the essential hop is a varietal property. Differences in the composition depends on the soil and weather conditions during the hops ripening. The aromatic hop varieties generally have a lower content than the essential bitter varieties.

Some of the aromatic components are of a particular importance, deciding not only on the scent of hops, but also affecting the bouquet of beer and depending on the variety of hop, they account for 60–80% of the total of the essential oils in the hops. One of these components is mircen. The characteristic feature of this substance is that it produces raw and base note of the fragrance in the beer, which is why the excessive amount of hops is undesirable. Moreover, such sesquiterpenes as humulene, β-caryophyllene and β-farnesene belong to those essential oils which have a beneficial impact on the smell of hops.

An important quality feature of hops is the ratio of the percentage of humulene (positive) to mircen (adverse). The share of these compounds is characteristic for different hop varieties. It is difficult to assess the impact of individual components on the characteristic smell of hops, as they all together form an unique ‘smell-taste’ bouquet [5].

Underappreciated group of compounds present in hops are polyphenolic compounds, whose main representative is xanthohumol (prenylated chalcone, Fig. 5), which has a wide spectrum of pharmacological activity, including, for instance, antioxidant property. It has also anticancer property (especially against breast carcinoma cells, colon, ovarian, prostate, leukemia) as well as antiviral one (including anti-HIV-1). Moreover, it has an influence on the parasites of the Plasmodium family, which causes malaria and additionally, it inhibits the low molecular weight lipids and has antidiabetic property by inhibition of lipid and glucose metabolism and can also function as a neutralizer of free radicals in human organisms. The xanthohumol represents 0.1 to 1.0% of dry weight of strobiles and its content depends not only on a hop variety but also on the weather conditions during the growing season.

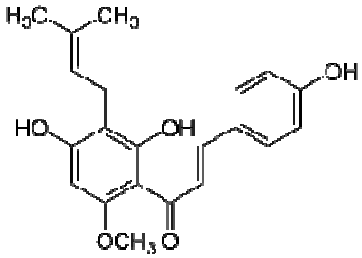


Fig. 5. The structural formula of xanthohumol

Yamaguchi and the research team applied in *in vitro* conditions ORAC method (Oxygen Radical Absorbance Capacity – the capacity to absorb free radicals), which is used to assess the antioxidant capacity in biological samples and food. The reference material (with the highest ORAC value of the edible plants) was polyphenol 60, containing greet tea catechins. Total ORAC value for xanthohumol was comparable to this of polyphenol 60 and significantly higher than that of vitamin E and vitamin C [6].

Table 3. ORAC value for selected chemical compounds present in hops strobilus

Compound	Total ORAC value (µmol of trolox)
Humulones	1,20
Lupulones	1,90
Xanthohumol	4,20
Vitamin C	1,40
Vitamin E	0,75
Polifenon 60	4,20

In order to obtain valuable compounds in lupulin, the extraction methods are performed.

Extraction:

– method of separating liquid or solid mixtures,
– is based on the separation of one or more components from the extracted phase by means of appropriate solvents,
– solvent should selectively dissolve the analyte,
– this method uses a large difference in solubility of the analyte in the extracted solution and solvent,
– extraction of a compound from one liquid phase to another is a process for determining the balance, dependent on the solubility of the compound in both solvents.

The ratio of the concentration of the one solvent to the second one is called the partition coefficient. It is a constant value at a particular temperature, characteristic for that substance and specific pair of solvents

This law, called the law of Nernst is expressed by the following formula:

$$\frac{c_A}{c_B} = constans = K$$

where: c_A and c_B stands for the concentration of the substance in the layers A and B, K – partition coefficient.

Approximately, partition coefficient is equal to the ratio of the particular substance solubility in both solvents.

The extraction with the liquids in the supercritical state is an alternative to conventional liquid extraction methods with organic solvents [7]. The ability of the solvent dissolution increases with the increase of pressure and at the pressure above the intersection of isotherm also the temperature. This phenomenon allows to increase the selectivity of the procedure by a simple manipulation of the extraction conditions.

The production of the extract with desired characteristics, together with the minimization of the productions costs, forces the proper selection of process variables. The classic approach to design technologies requires significant funding and time. Therefore, the significant development of techniques from the border of mathematics and statistics is observed. It is done through the use of planning experiment methods (DoE) and optimization methods of Response Surface Methodology (RSM). This approach allows the identification of the input and output variables of a test process but also the experiment planning on the basis of the specific mathematical rules, which ensures the achievement of the maximum amount of the information with the least amount of researches. The results of the research allow to develop a mathematical model of the process and its optimization in order to achieve desired final result [8]. One of the most important criteria of the final product is the right composition with the minimized manufacturing costs.

Carbon dioxide is widely used in the food industry due to its characteristics. It is characterized by the low viscosity, high diffusivity, a relatively low critical parameters (304.2 K and 7.38 MPa), the lack of corrosiveness, non-toxicity as well as non-flammability. Furthermore, it is characterized also by high volatility, which facilitates its removal from the product after the extraction process. From a physiological point of view, it is harmless and inexpensive as it is an air component [9].

The extraction of lupulin with the use of a supercritical carbon dioxide in order to determine the efficiency of the process and the quality of the products was performed in The Supercritical Extraction Department of Institute of New Chemical Synthesis. Comparatively to the supercritical extraction of the lupulin, the extraction using a Soxhlet apparatus with n-hexane as a solvent was performed.

2. EXPERIMENTAL PART

2.1. Materials and research methods

The material for the research was lupulin coming from the pipe pneumatic conveying of the plant used for drying hops (the year of harvest cones – 2015).

Fig. 6. Lupulin

During the pneumatic conveying of the hop cones the partial loss of lupulin is observed, which is caused by the lupulin falling from the cones and filling the pipelines.

Losses of drying

The loss of drying the material which is to be extracted was determined by the drying method with the use of the weighting dryer Radwag MAC 50/1. Tests were performed in triplicate and the results are shown with the standard deviation.

Table 4. Loss of drying

The measurement	Moisture % wt.
1	5,50 ± 0,01
2	5,49 ± 0,01
3	5,51 ± 0,01
Average value: 5,50 ± 0,01 % wt.	

Particle size analysis

Particle size distribution of the lupulin is shown in Figure 7.

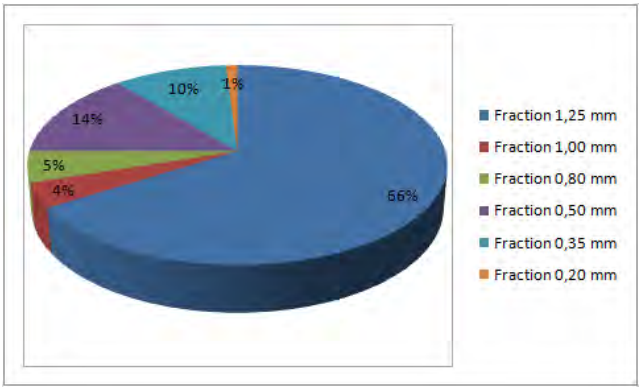


Fig. 7. The particle size distribution for the lupulin

To study the extraction process in a solid-supercritical liquid and two-stage separation the universal plant by SITEC (SITEC-Sieber Englineering AG Switzerland) was applied, which is located in the Supercritical Extraction Department of the Institute of New Chemical Synthesis.

The plant laboratory is equipped with the extractor with the capacity of 1 dm^3 heated by the oil heating jacket and two separators. The parameters of the plant are as follows extraction temperature up to 200°C and the extraction pressure up to 1000 bar. The circulation of carbon dioxide is controlled by the diaphragm pump by Lew with a capacity of 10 liters by hour. The apparatus is presented in the Figure 8.

Fig. 8. The laboratory plant of solid-supercritical carbon dioxide extraction

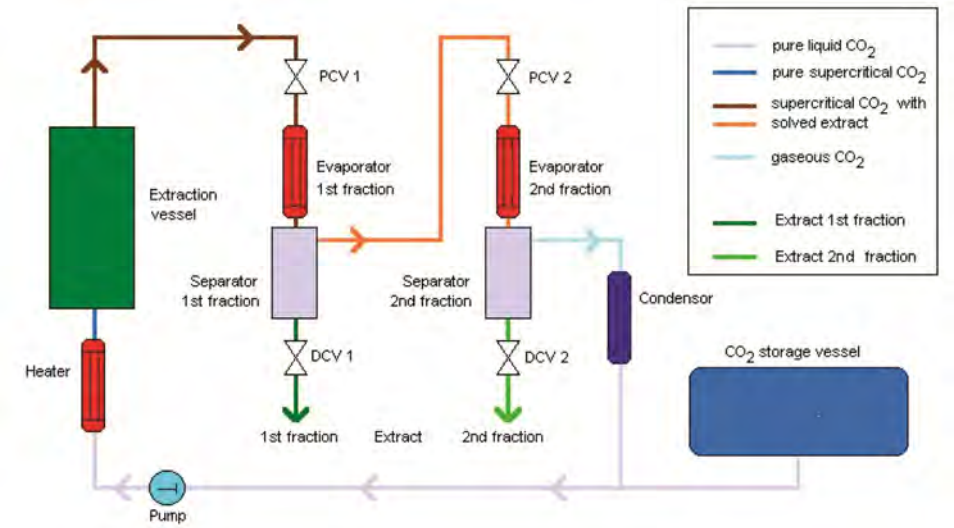


Fig. 9. Technological diagram of an plant laboratory with two-stage separation for plant materials extraction using supercritical CO_2

The research on the lupulin extraction process with the use of the supercritical carbon dioxide was performed under the pressure of 150–350 bar and the temperature of 40–60°C. The pressure of the first stage separation was 100 bar whereas the pressure of the second stage separation was 54 bar. The temperature of the first stage separation was 60°C and 35°C in the case of the second stage separation. The flow rate of carbon dioxide during the carried experiments was 8 kg/h. The extracts obtained on the research plant were chromatographically analyzed. For this purpose, the liquid chromatograph ACCELA 1200 Thermo equipped with UV-Vis detector (U-HPLC) was used. Chromatographic separation of the compounds was carried out at the temperature 35°C on Hypersil Gold column with the dimensions of 200 x 2.1 mm. Both acetonitrile and water were used as the components of the mobile phase. The analysis was performed under gradient elution conditions (Table 5).

Table 5. Gradient conditions

Time (min.)	% A	% B
0	40	60
14	40	60
18	16	84
28	40	60
30	40	60

A: water + 0,1% TFA

B: ACN + 0,1% TFA

The flow rate of the analysis was 0.40 ml with 2µl as the injection volume. The identification and peak measurement was conducted at the wavelength of 314 nm. The chromatogram of the extract obtained from the lupulin in presented in Figure 10.

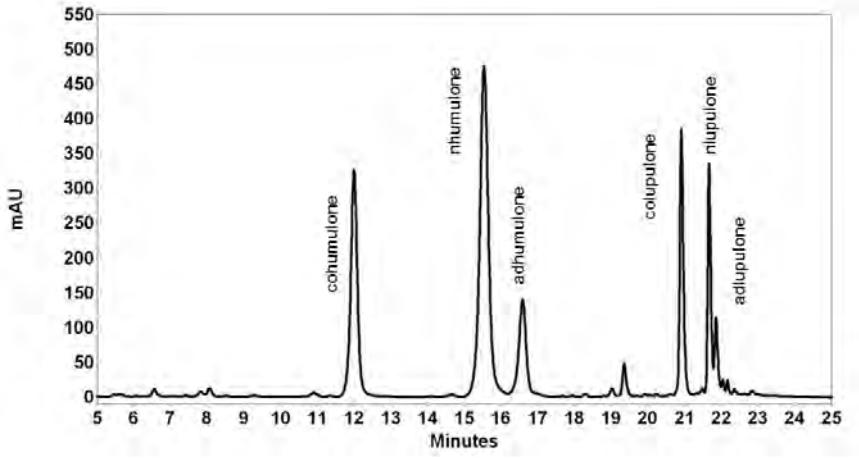


Fig. 10. The chromatogram of the extract obtained from the lupulin

Comparatively to the supercritical extraction of the lupulin, the extraction with the use of Soxhlet apparatus with n-hexane as a solvent was performed. Soxhlet extraction involves placing approximately 5 g of the material in the core and extracting it with the use of 300 ml of n-hexane, which was already in a flask and then was brought to the boil. The resulting solvent vapors are condensed in the cooler. When the condensed solvent flows into the cup, the active compounds contained in the extracted material are extracted. When the level of a liquid solvent reached the higher level than so called trap, it was then automatically drained back to the flask. During the extraction, the solvent changed into green as it contacted with the material (Figure 11). The extraction was carried out until the pure distillate was obtained.

Fig. 11. SoxE1 n-hexan extract

2.2. Discussion of the results

The results of the lupulin extraction using supercritical carbon dioxide are shown in Table 6 and 7.

Table 6. The results of the lupulin extraction

Exp. No./ Separator No.	Pressure	Temperature	Efficiency	α-acids	β-acids	∑ α- and β-acids
	bar	°C	%	% wt.	% wt.	% wt.
Ex1/separator 1	150	40	41,15	40,1	21,0	61,1
Ex1/separator 2				52,3	22,8	75,1
Ex2/separator 1	150	50	38,94	51,6	23,0	74,6
Ex2/separator 2				41,6	22,2	63,8
Ex3/separator 1	150	60	36,68	57,0	27,7	84,7
Ex3/separator 2				37,1	21,0	58,1
Ex4/separator 1	250	40	48,74	49,1	22,1	71,2
Ex4/separator 2				45,3	22,7	68,0
Ex5/separator 1	250	50	47,88	48,1	19,7	67,8
Ex5/separator 2				47,1	21,4	68,5
Ex6/separator 1	250	60	49,23	46,5	20,8	67,3
Ex6/separator 2				44,4	23,3	67,7
Ex7/separator 1	350	40	45,18	46,3	19,7	66,0
Ex7/separator 2				47,6	22,7	70,3
Ex8/separator 1	350	50	51,38	49,0	20,2	69,2
Ex8/separator 2				47,1	23,0	70,1
Ex9 separator 1	350	60	50,35	44,1	18,1	62,2
Ex9/separator 2				46,8	21,9	68,7

Table 7. The contents of α- and β-acids in the lupulin extraction residue

Exp. No.	α-acids	β-acids	∑ α- and β-acids
	% m/m	% m/m	% m/m
Ex1	3,89	0,70	4,59
Ex2	3,67	0,75	4,42
Ex3	4,81	0,96	5,77
Ex4	2,16	0,46	2,62
Ex5	2,87	0,52	3,39
Ex6	3,28	0,55	3,83
Ex7	2,00	0,38	2,38
Ex8	1,55	0,25	1,80
Ex9	0,56	0,07	0,63

The operating conditions of Soxhlet extraction method and the results of the extraction are presented in Table 8.

Table 8. The yield of the extraction with n-hexane

Exp. No.	Mass of raw material g	Time of extraction h	Volume of solvent ml	Mass of extract g	Residue g	Extraction yield % wt.
SoxE1	5,64	5	300	2,88	2,74	51,06
SoxE2	4,69			2,33	2,36	49,68
SoxE3	4,98			2,60	2,33	52,20

The average yield of the lupulin Soxhlet extraction was 50.98% and was comparable to the yield of the supercritical extraction under the pressure of 350 bar and the temperature of 50°C, which was 51.38%. However, the time of the lupulin extraction with Soxhlet techniques was significantly longer (approximately two and a half times). Figure 12 presents the appearance of the residue after lupulin extraction with Soxhlet technique.

Fig. 12. The residue from SoxE1 extraction

On the basis of the obtained results of the experiments, the mathematical model of the extraction process was developed with the statistical analysis of the resulting model. The model defining the extraction yield is described by the following equation:

$$Y1 = b_0 + b_1X_1 + b_2X_1X_1 + b_3X_1X_2,$$

where: b_i – is the equation coefficient,
X_i – encoded value of the process variable.

The correlation coefficients obtained with the level of significance are presented in Table 9.

Table 9. The correlation coefficients of the developed mathematical model

Equation coefficient	Coefficient	P value	Std Error	-95%	95%	t Stat
b0	47,99	>0,0001	0,501	46,89	49,09	95,87
b1	5,343	>0,0001	0,468	4,312	6,373	11,41
b2	-3,939	0,0001	0,685	-5,447	-2,430	-5,746
b3	2,410	0,0039	0,662	0,953	3,867	3,639

The results were used in order to construct the response surface. Figure 13 shows the resulting response surface.

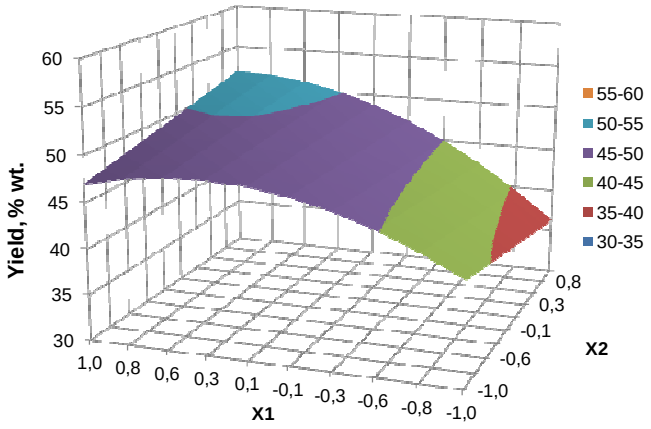


Fig. 13. The extraction yield (Y) depending on the variable X1 (extraction pressure) and X2 (extraction temperature)

Table 10 shows the results of the statistical model.

Table 10. Statistical analysis model

Parameter	Value
|R| – correlation coefficient	0,970
R2 – coefficient of determination	0,941
R2 adjusted	0,925
R2 for prediction	0,868

For the developed model, a high determination coefficient (R2) was achieved, which informs that the model explains approximately 94% of the original variation of the analyzed Y1 function. The determination coefficient R2 is in good agreement with the adjusted R2. The difference between the adjusted

R2 and predicted R2 is less that 0.1, therefore the model can be used to move in the projected variable space. The relationship between the predicted yield of the supercritical extraction of the lupulin according to the developed model and the experimental data is shown in Figure 14.

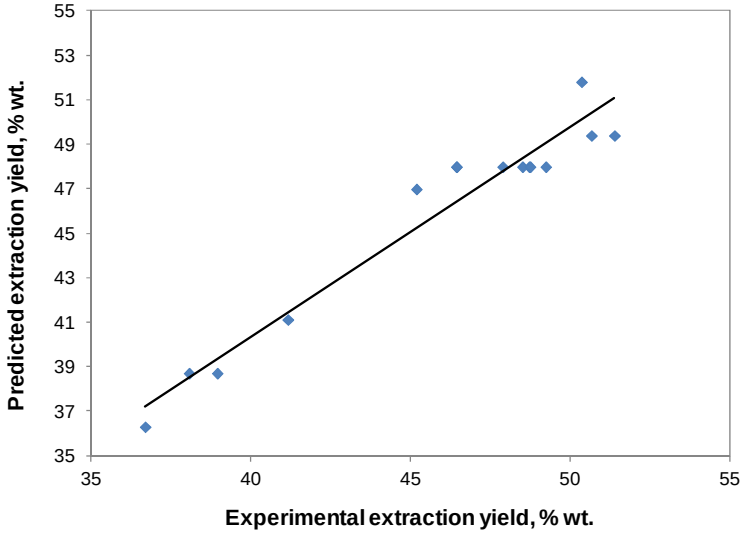


Fig. 14. The precision of the developed mathematical model for the lupulin supercritical extraction yield

In Table 11 the results of ANOVA analysis are shown.

Table 11. ANOVA analysis

ANOVA						
Source	SS	SS%	MS	F	F Signif	df
Regression	309,48	94	103,16	58,81	4,63841E-07	3
Residual	19,29	6	1,754			11
LOF Error	10,89	3 (56)	3,630	3,4553	0,07141	3
Pure Error	8,404	3 (44)	1,051			8
Total	328,78	100				14

The results confirm that the mathematical model is statistically significant (Fisher-Snedecor test = 58.81, p <0, 0001). The error lack of the fit is statistically insignificant, so there is little chance that the course of the function is caused by the noise.

2.3. Conclusions

The study confirmed the hypothesis that the lupulin located in the plant pipelines used for drying hops is a valuable biological material, in which, despite the mechanical damage, there are many important bioactive compounds, especially α-acids.

The use of spectral detector combined with liquid chromatography allowed to control the supercritical extraction of lupulin as residues in the pipelines of the cones granulation plant.

The highest yield was obtained for lupulin supercritical extraction under the pressure of 350 bar (51.38%) and the temperature of 50°C and it was comparable to the yield obtained for Soxhlet extraction. Under these conditions, the high concentrations of α-acids in extracts were achieved.

Statistical analysis of the resulting model, which showed that the most significant effect on the yield of the lupulin extraction process has both temperature and pressure as well as interactions between these two parameters.

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THE ECONOMIC ASPECTS OF NATURAL RAW MATERIALS EXTRACTION WITH SUPERCRITICAL CARBON DIOXIDE

Summary

Economic aspect of the production of plant extracts using supercritical extraction plays a very important role and decides about industrial applications. The technique of supercritical extraction generally is considered to be expensive, but the calculation of the manufacturing extracts cost using this technique does not support this thesis. In this chapter the methodology for calculating the cost of manufacturing the plant extracts using supercritical carbon dioxide technology was presented. The proposed methodology can be used to calculate the cost of preparing extracts in existing industrial or pilot plants. It can also be used to design new plants.

1. INTRODUCTION

Natural organic raw materials of plant origin are one of the richest sources of many remarkably valuable substances especially for food, pharmaceutical and cosmetic industries. The extraction of raw materials with supercritical carbon dioxide at the pressure above 7,38 MPa and the temperature above 33,1°C is one of the fastest developing field of research and commercial uses in the last forty years.

Carbon dioxide in supercritical state especially at the pressure of 20–50 MPa and the temperature of 40–80^{o}C is a solvent of choice for extracting different types of compounds, such as esters, alcohols, aldehydes, ketones, volatile oils, aromas, mono- and sesquiterpenes, and less good for extracting waxes and polyphenols due to their polar nature. Supercritical carbon dioxide has not been applied for extracting carbohydrates, fruit acids, starch, proteins, mineral salts and glucosides because their solubility is very low.

There are already known hundreds of plant extracts obtained by this method of extraction. These extracts are generally a mixture of different chemical compounds, a part of them with valuable properties, and the remaining ones representing an inert admixture or undesirable matter.

The review of the literature revealed the great interest in the extraction of algae, which was considered as a prospective raw material to obtain oils as biofuels and natural colors for food (chlorophylls and carotenoids). One of the greatest and the longest working industries is the production of hop extract for beer. In addition, the supercritical carbon dioxide extraction found its application in removing caffeine from coffee beans and tea leaves. Cosmetic industry has been provided mainly with oils and essential oils obtained by $ScCO_2$ extraction techniques, while the pharmaceutical industry has been demanding the antioxidants (flavonoids, polyphenols, tocopherols, carotenoids), known for their different and broad pharmacological activities. As far as food industry is concerned, the most important are different oils and natural colors (carotenoids, chlorophylls), spices (pepper as a spice, pepper as a vegetable, garlic, onion, ginger), herbs (mint, rosemary, oregano, thyme) and antioxidants. Plant extracts might be used as pesticides and fungicides, for instance, nicotine from tobacco, α- and β-acids from the hop, as well. Recently, the numerous literature on natural extracts and extraction applying the supercritical carbon dioxide have been published [2–4].

2. ECONOMIC ANALYSIS OF THE RAW MATERIAL PROCESSING

As it is known, the basic aim of the service or production activity of each company is the efficient management combined with the technical and economic efficiency. To keep the efficiency at a desired level it is necessary to conduct periodic Economic Analyses (EA) of the current and anticipated activity with reference to changing conditions. EA constitutes a set of requested and processed pieces of information of a technical and economic nature, which concern a specific extraction process (product, raw material, waste, process, plant and costs). The main and final aim of EA is to specify and define all unit costs of product manufacturing or raw material processing and their sum but also the share of particular unit costs in total, that is the structure.

According to economical nomenclature, the total cost of product manufacturing or raw material processing is called the cost of manufacturing (COM) and includes:

- direct costs (materials, energy factors, labor and all other direct costs),
- departmental costs,
- general overhead costs,
- the costs of sale.

Direct costs should cover all unit costs that can be distinguished and assigned to a particular product or raw material. In case of the absence of such a possibility they should increase departmental or general overhead costs. The correct COM calculations should prevent accidental mistakes and in fact its lowering.

In the available literature only simplified methods of determining COM of certain extracts and optimization of certain extraction parameters were found

[1–17]. However, the comprehensive discussion of EA and the presentation of an easy method of COM calculation have not been presented.

From the practice and literature it is known that COM may be applied to the mass or the volume of the manufactured product or the mass or the volume of the processed, that is extracted raw material.

COM is the basic criterion for assessing the effectiveness of an economic activity concerning the production of a known or a new extract or the processing of a known or a new raw material in actual or anticipated conditions in an existing or a new plant. COM calculations may be a basis for the valuation of the extraction service in an existing plant or for making the decision concerning the purpose of building and the size of a new extraction plant. COM variant calculations allow to determine the impact of the various parameters on its size and the structure.

3. THE SCOPE AND THE METHOD OF ANALYSIS

For the proper and responsible conduct and correct interpretation of EA the knowledge (information) of an economic and technical nature but also production organization is required. Only full knowledge enables taking into consideration all factors which generate costs, however, on one hand incomplete knowledge causes the need to adopt assumptions which reduce the probability to obtain correct and reliable results, but on the other hand they increase the risk of making wrong decisions.

Presented report specifies and discusses the scope of requested and processed pieces of information which constitute a complete EA of extraction. In order to facilitate completing and using the information they have been put together in the table below.

The knowledge included in EA should contain possibly the most accurate information concerning:

- products,
- raw materials,
- extraction residues,
- extraction process,
- extraction plant,
- cost of manufacturing (COM) of the product or raw material processing.

3.1. Data about the product

The information about the product concerns mainly the scale of the production but also properties and factors which may generate the costs not connected directly with the extraction process. As it is known, the scale of production is connected closely with the size and a number of plants of extraction (extractors), the number of personnel, the size of warehouse space, the demand of the raw material, energy media, etc. The one fact should be taken into

consideration that the size of the production may be limited by the availability of the raw material or the possibility of product disposal.

The form of the product (liquid of low viscosity, liquid of high viscosity, paste, powder) and the specific mass may have a significant influence on the way and the cost of emptying the separator but also on the way and the cost of packing.

Other properties of the product, such as, for instance, toxicity, volatility, combustibility, persistence, temperature sensitivity, moisture, light, oxygen can cause the necessity for additional equipment, and the security of the plant, the warehouse and the service. The properties of the product may have the influence on the need for approval on its production, the need for insurance but also on the way and the costs of managing (utilization) in the event of its inadequate quality and the way and the costs of cleaning the plant.

The requirements concerning chemical composition can limit the time of the extraction, causing the need to possess additional devices and to incur the labor input connected with the standardization and quality control.

The requirements concerning the product packaging can cause the necessity to ensure the appropriate devices and additional service.

The information about an actual and anticipated market price of the same and similar product helps to assess the competitiveness and possible profit.

3.2. Data on raw materials

Information about the raw material concerns mainly the size of its demand or the availability in the market but also the properties and factors, which can generate the costs not connected directly with the extraction process. As it is known, the demand for the raw material is closely related to the size of warehouse place and the internal transport.

The volume of the raw materials divided by the volume of the feedstock to extractor constitutes the number of charges of the extraction. However, it should be taken into consideration that the majority of the organic materials in the extraction significantly increases own volume and that is why the volume of the fresh feedstock in the extractor or basket should constitute 70–80% of its capacity.

The form and the specific mass of the raw material may have significant influence on the occupied warehouse space as well as on whether the preparation of the material to the extractor is required. The aim of the material preparation for the extraction is mainly to facilitate the access of carbon dioxide to extracted substances and may rely on crumbling (milling, cutting, breaking), sieving, squashing, flaking, granulating, macerating, etc. There are the cases when the raw material requires drying in order to lower excessive humidity, which disturbs the process of extraction. The choice of the method of the raw material preparation may be based on the knowledge, own research or by the analogy to similar materials. The preparation of the raw material involves the necessity to have appropriate equipment, time and costs. In EA the management or utilization of packagings remaining after the raw material or the possible insurance of the raw material should also be taken into consideration.

The content of the main extracted substances in the raw material has crucial influence on a yield (productivity) and the quality of the extract (product). Since the quality of the material often depends on the conditions and time of its acquisition and on the storage conditions it is necessary to specify and control the requirements in this area. The quality control of the raw material is connected with the necessity for sampling, performing analysis and bearing the costs related to this.

Such physicochemical properties as dusting, hygroscopicity, toxicity, combustibility, shelf life, sensitivity to light, temperature, moisture may cause the need to provide special conditions for storage, preparation for extraction, transport and service protection. It should also be taken into consideration that some of the raw materials or substances contained in them can be allergens and can cause allergic reactions to people who have contact with them.

To conduct a full EA it is necessary to know current and anticipated prices of the raw material among different suppliers.

3.3. Data on the extraction residues

The information about the residues after extraction defines its properties but also the method and the costs of the quality control, storage, sale or utilization. These pieces of information are important especially in the case of a large mass (volume) of the residue. The knowledge about the properties of extraction residues gives the opportunity to find the most beneficial method of usage or utilization. Some of the extraction residues may be used, for instance, for the production of animal feed but also as a compost or the organic fertilizers.

3.4. Data on the extraction process

The information about the extraction process is a set of data of which the source is:

a) a project of the process of an existing extraction plant or
b) a set of data of the project of the process of a new extraction plant or
c) a set of data of the extraction of new raw materials in an existing plant.

They include all main process parameters and consumption factors but also the price of all media, energy and labor. Parameters and factors concern only the process usually defined in the plant terminology as the effective extraction pressure and the capacity of the extractors, for instance, the extraction plant parameters listed as follows: pressure at 400 bar, volume – 500 liters. The process based on a different scale, pressure, extraction time and the method of plant operation will be characterized by other parameters and consumption factors of the raw material, materials, energy and labor.

The exemplary consumption factors of energy and carbon dioxide during one working hour in the plant with the production capacity of 200–1500 kilo of the raw material per day, at the pressure up to 300 bar and the temperature up to 80^{o}C with 3 extractors and 2 separators are provided in the publication [7]:

- electricity 600 kWh
- cooling water 3 m^3
- low pressure steam 150 kg/h
- carbon dioxide 30 kg/h

The consumption of carbon dioxide is composed of three losses associated with a) removing air from the plant before starting the extraction, b) reducing pressure in circulation from the condensation pressure (55–65 bar) to atmospheric pressure at the end of each cycle c) replacing contaminated CO_2 which is recommended after each change of the extracted raw material. Small losses may occur during dehydration of liquid CO_2 or by incidental leaks in the plant. Total consumption of CO_2 is 1–2% of the mass pumped by the pump [9, 10, 13].

The prices of energy media and carbon dioxide according to different sources

Factor	[1] US $/GJ	[6] US $	[7] €	[8] US $/GJ	[9,10] US $	INS US $
Steam	3.18	-	0.05 / kg	2.39	4.20 / t	8.75 / GJ
Cooling water	19.99	-	1 / t	19.11	0.19 / t	1 / t
Electricity	16.79	0.02 / MJ	0.1 / kWh	16.72	0.092 / kWh	0.1 / kWh
CO_2	-	0.3 / kg	0.6 / kg	-	-	0.075

A very important information about the extraction process is the dependence of the extraction, that is efficiency and product quality on the extraction time at a determined flow rate of CO_2. As it is known from the practice and literature, in the final phase of extraction the amount of obtained extract in time unit is decreasing faster and faster, the composition is changing and at some point the increase in the value of obtained extract does not cover the increase in the costs. Extending the extraction time becomes uneconomic, although for different reasons, for instance, on the property of the extraction residues, it may be intentional.

3.5. Data on the extraction plant

The information about the extraction plant covers technical characteristic of the main apparatus and devices, specification of auxiliary equipment as well as the information about the way and the costs of exploitation. They can concern an existing and designed plant. In the event of an existing plant all information is known, however in the case of designed information these are the assumptions and alternatively a data provided by the potential suppliers.

The name of the plant should include the most essential information which enables decision on the operational capability and the costs of the plant, which are the extraction pressure and the extractor capacity. For these parameters the CO_2 pomp and eventually the pomp of the entrainer is adjusted. While selecting the pump capacity and the extraction time is often assumed that for a particular raw material the mass ratio of the CO_2 pumped through the raw material to the

mass of the raw material is constant. The number, capacity and operating pressure of the separators must be chosen according to extraction process data.

The information about the cost of a new plant can be provided by the potential suppliers or it can be evaluated on the basis of the literature data. The main European suppliers of the extraction plant with carbon dioxide are such companies as UHDE/Germany, SITEC/Switzerland and NATEX/Austria. For instance, NATEX offers standard plant with 1, 2 or 3 extractors, the capacity of which is 200 and 300 liters, at the pressure up to 70 MPa and the extractors, the capacity of which is 600, 800, 1250, 2500 and 3500, at the pressure up to 55 MPa.

The presentation of I. Kikic from the University of Trieste can be helpful in evaluating the cost of the plant presenting the dependence of the investment cost on the number and the capacity of extractors working at the pressure of 550 bar with the CO_2 flow rate of 15 kilogram per hour. The cost of the investment in millions of Euro in 2007 is presented by the formula:

a) 1 extractor with the capacity of V = 200 – 600 liters — $y = 0.8159\ln(V) - 3.0964$

b) 2 extractors with the capacity of V = 400 – 1200 liters — $y = 0.9167\ln(V) - 4.0866$

c) 3 extractors with the capacity of V = 600 – 1800 liters — $y = 1.0163\ln(V) - 4.9147$

The number of extractors can also affect the way of their exploitation and the efficient use of the operating system. If there are two extractors in the plant it is possible to extract in one of them, while at the same time the second can be emptying and again loading with the raw material. If there are three extractors in the plant it is possible to extract in one of them and at the same time second can be emptying and the third one can be filled with the raw material.

The examples of the investments in the extractor plants with two extractors and two separators depending on the capacity of the extractors were presented by J.A.R. Uribe et al. in 2009 [13], J.M. Prado et al. in 2011 [9] and R.N. Cavancanti et al. in 2012 [10].

Capacity, dm^3	**Cost, thousands of US $**		
	according to [13]	[9]	[10]
5	75	100	-
12	271	-	-
50	400	300	-
100	-	-	300
200	1253	-	-
400	2000	-	450
500		1150	
1000			850

On the basis of the data provided by Uribe the correlation $y = 31901V^{0,6909}$ was presented.

The publication [16] included the cost of the plant with two extractors with a capacity of 600 liters in the amount of 2,3 million Euros. The publication provides that the cost of the extraction plant is proportional to the capacity of facilities of the raw material to the power of 0,6 or more accurately to the capacity of the extractors and the flow rate of CO_2 to the power of 0,24. The comparison of the above mentioned literature data concerning the dependence of the plant costs on its size shows a big divergence.

The prices of individual devices, in thousands of Euros, in the extraction plant with the capacity of the facilities of the raw material of 200–1500 kilo per day (the capacity of extractors is not specified) at the pressure up to 300 bar and the temperature up to 80^{o}C with 3 extractors and 2 separators, CO_2 flow rate of 1500 kilo per hour, were presented in L. Casas's publication:

Extractors	1200
Baskets	28
Separators	93
Containers	379
Heat exchanger	107
Pumps and compressors	380
Support facilities	52
Total direct costs	2239
Total indirect costs	895.6
Total fixed costs	3134.6

The plant was designed for the extraction of grapes skin, olives and sunflower leaves but also microalgae.

The cost of the extractors of the same capacity depends on the ratio of their height to diameter, which is typically in the range of 3–9 and depends mainly on the particle size of the extracted raw material. For small particles and aerated material this ratio is 3, for the particles of 0.4–0.8 mm – about 6 and for big particles, like, for instance, coffee beans – up to 9. The larger diameter of the extractor the greater the thickness of its walls, higher closure and higher price.

In addition to the investment costs associated directly with extraction plant the costs of the ancillary equipment of the department should be taken into consideration in EA. It may be, for instance, comminutors, sifters, grinding machines, wages, granulators, driers, homogenizers, vacuum cleaners, pressure washers, packing machines, hoists, trolleys, systems of pneumatic transport, filtration ventilation and/or air conditioning. Their size and technical characteristic should be appropriate to the size of the extraction plant. In the event of own quality control of the raw material, products and extraction residues an appropriate laboratory equipment will be also necessary.

The relevant information about the plant, which may have crucial impact on its operating costs, is the way of loading and unloading extractors, the way of emptying separators and the way of cleaning it. If the plant is or is to be used for different raw materials extraction, after the extraction of each raw material, the

plant should be precisely cleaned, so that the extraction residues from the previous raw material do not contaminate the extract of a new raw material. The equipment which should be cleaned constitutes extractors, separators, heat exchangers, CO_2 container but also pipelines throughout the circulation of CO_2. To clean the interior of the extractors precise vacuuming is relevant. The way, time and the cost of cleaning the interior of the separators, pipelines and fitting depends mainly on the form of the remaining extracts in them. Sometimes only CO_2 circulation is enough, but sometimes the mechanical removal (e.g. scraping) or solvent cleaning (e.g. isopropanol) is necessary. As the liquid CO_2 in the capacitor container includes dissolved and not separated extract and water it is intentional to exchange CO_2 to fresh and clean. The costs associated with cleaning plants must be converted into unit of mass or volume of the raw material or the extract.

Depreciation, repairs and maintenance of plant and ancillary equipment of the department are usually the elements of departmental costs (indirect). The annual rate of fixed costs of the investment (FCI) of fixed assets is determined by relevant regulations and for apparatus and pumps it is 10–12.5%. The cost of repairs and maintenance is generally established as 2–4% of depreciation.

The way of operating the plant has a high impact on COM. Incomplete use of working time of the plant increases the unit cost of depreciation and other departmental costs but also consumption factors of energy in the refrigeration and heating circuit.

For security reasons, the number of people operating the plant during one shift should be the minimum of two, and because of the laborious loading and unloading extractors and emptying separators it is usually from 2 to 4.

3.6. The cost of manufacturing (COM) of the product or the raw material processing, net and gross selling price

It should be paid attention to all COM forming cost units to be converted to the same mass or volume of the raw material or the product. From the practice and literature it is known that COM and its structure to a large extent depends on the size of plant but also on the pressure and working system, that is and effective working time per year. In the event of small plants working at lower pressure and in one or two-shift system COM is high, but in the case of big plants working at higher pressure in three-shift system it is much lower. In COM structure with the increase of the plant size direct labor and indirect costs decreases, while the cost of the raw material, ancillary materials and energy increases. In the event of the extraction of the same raw material with the same parameters, the scale of plant does not have significant impact on unit costs of the raw material, materials and energy consumption.

Indirect departmental costs constitute a large part in COM. They may be even higher than direct costs. There are no general requirements, according to which direct and indirect costs should be classified and specified. The specification and the amount of direct costs are determined by the financial unit of the company,

usually as the multiplicity of direct costs. These include the costs of department upkeep usually, such as building and equipment depreciation, insurance, conservation, repairs, cleaning, internal transport, lighting, heating, ventilation, air-conditioning, drinking water, telephones, supervision pay, business trips, protective clothing, cleaning agents and external services. They are not included in direct costs. Indirect costs may include also the costs of upkeep of departmental laboratory. It is easy to determine those costs, if the department has only one plant. If there are more plants and if they are different and differently exploited, the issue of allocation of the part of departmental costs to each plant is most often discretionary according to adopted key which does not always correspond to the reality. For this reason, the authors of this study advocate for assigning to direct costs as much as possible the number of unit costs which can be assigned to a particular plant or the raw material or the product.

The operating costs belong also to indirect costs and they increase COM. The amount of those costs is determined by financial unit of the company, usually as the multiplicity of the technical cost of manufacturing. The operating costs include the costs of upkeep of management, administration and non-productive units (for instance, factory laboratory, book keeping, planning, supply, stores, transport). The issue of allocations of the part of the departmental costs to each department, each plant of the raw material or the product is discretionary according to adopted key similar as in the case of departmental costs.

Probably due to departmental and factory costs in Turton's COM formula a numerical factor at depreciation is 0.304 instead of 0.1 and at direct labor is 2.73 instead of 1.

The examples of COM in € / kg of the raw material are listed below:

- isobaric decaffeination of coffee beans [5] 0.55 – 0.85
- non-isobaric decaffeination of coffee beans [5] 0.75 – 1.1
- the removal of pesticides from gingseng [5] 5 – 14
- aromatic raw materials [16] 3 – 8

The examples of minimum of COM in US$ / kg of the extract are listed below:

- clove (dianthus) [1, 9] 9.15
- cloves [13] 4.70
- ginger [1] 99.80
- Buriti palm [8] 22.81
- Pupunha palm [8] 17.15
- PPF (press palm fiber) 19.46
- pomergranate leaves [10] 114.36
- Habanero Pepper [13,15] 540.19

The exemplary analysis of COM dependence on the pressure (10, 15, 20 and 30 MPa) and the temperature of the extraction (40 and 50^{o}C) but also the capacity of the extractors (0.1; 0.4 and 1.0 m^{3}) for the extraction of pomegranate leaves is presented by R.N. Cavancanti et al. [10] using the formula of Turton's et al. [1, 18]:

$$\mathbf{COM = 0.304\ FCI + 2.73\ COL + 1.23\ (CRM + CWT + CUT)}$$

where:

- FCI – fixed cost of investment
- COL – cost of operational labor
- CRM – cost of raw material
- CWT – cost of waste treatment
- CUT – cost of waste utilization (extraction residues)

This analysis confirms that COM decreases in a high extent with the increase of the extraction capacity and the pressure and in a lower extent with the increase of extraction temperature.

In the case of services provision of the raw material extraction which is the property of the principal COM is reduced by the costs incurred by the principal. Possible revenue from the sales of the waste can decrease COM and reduce costs incurred by the principal.

Comparing the calculated COM with COM or market price of the same or similar product with literature data allows to assess analyzed project, whether it is and to what extent competitive and economically justified. This comparison allows also to estimate possible profit. It is generally assumed that the profit should be at least 3 % of technical cost of manufacturing.

To determine net selling price (for those who are VAT payers) the cost of manufacturing should be increased by estimated profit.

In order to determine gross selling price (for those who are not VAT payers) the net price should be increased by VAT (currently 23%).

The structure of COM shows what influence on this cost have particular unit costs, which of them are crucial and in which it is worth looking for savings, as well as those which have small secondary importance.

3.7. Case study

It has been admitted that basic calculations will be done for one charge, that is the extraction of 800 kg of the raw material in two separators within 12 h (the extraction within 8 hours and loading and unloading within 4 hours), in which 24 kg of the product is obtained.

The processing of the whole amount of the raw material is 50 tones, what is equivalent to 62.5 charges (the last charge in extractor), the time of which will be 756 h (31.5 days) + 2 days on plant cleaning. The total time of plant exploitation is 33.5 days.

In the presented example in the cost of manufacturing, direct costs are slightly over 45%, departmental costs – almost 46% and general costs – slightly over 9%.

In direct cost the basic position (60.4%) is electricity and then heating steam (17.9%) and direct labor (15.8%). Total cost of other direct costs is only 5.9%.

In departmental costs the basic position (almost 69.9%) is depreciation of fixed assets. The share of conservation and repairs is 19.4% while the other departmental cost is about 10.7%.

In the cost of manufacturing the basic are:

- depreciation : almost 32%
- electricity : slighty over 27%
- departmental costs : slighty over 9%
- maintenance and repairs : almost 9%
- heating steam : slighty over 8%
- direct labor : slighty over 7%
- other departmental costs : almost 5%.

Total of the other costs is slightly over 2.6%.

A unit extract price is a result of the above discussed component costs and is strongly dependent on the capacity of the plant. In Fig. 1 there is a graph of the cost of manufacturing and sale price as a function of extraction yield. Such a graph like this can be made for each existing plant. Of course, the calculated unit prices may be different for each particular country as the particular cost compounds are usually also different.

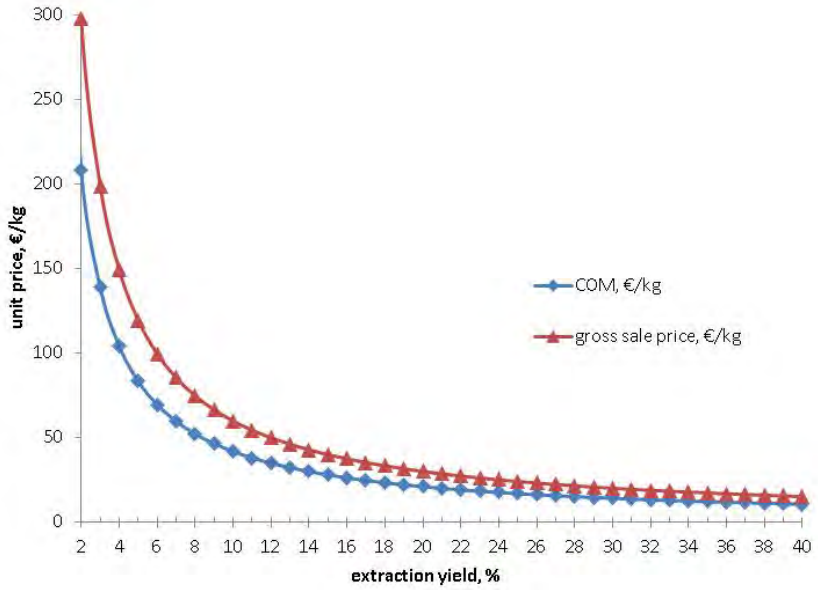

Fig. 1. Simulated unit price of extract vs. extraction yield for an extraction plant

SUMMARY

Economic analysis is very important factor at every stage of design and exploitation of a new or existing extraction plant.

A brief description of a method of calculating the cost of manufacturing of extract with supercritical technology and the cost of sales of the extract was presented. The proposed methodology is universal and can be applied to existing plants and newly designed ones. It can also be used to calculate the cost of services on specific research or industrial plants.

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